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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The key transcriptional regulators of the cellular hypoxic response, Hypoxia Inducible Factor-1 (HIF-1) and NF-κB, are responsible for induction of genes that regulate anaerobic metabolism, angiogenesis and cell survival. We hypothesized that cancer cells subvert these normal hypoxia-dependent mechanisms to enable their own deregulated survival and growth. Our results indicate that loss of the *p53* tumor suppressor gene augments HIF-1- and NF-κB-dependent transcriptional activation of the vascular endothelial growth factor (*VEGF*) gene and contributes to the angiogenic switch during tumorigenesis. In addition, we find that activation of NF-κB by HER-2/neu- and IGF-1 protects breast cancer cells from hypoxia- or death receptor-induced apoptosis. Conversely, repression of NF-κB by inhibition of IκB kinase (IKK) and casein kinase II (CK2) sensitizes breast cancer cells to hypoxia- or Apo2L/TRAIL-induced death. Together, our studies indicate that the constitutive activation of HIF-1 and NF-κB in breast cancers may underlie their angiogenic and apoptosis-resistant phenotype; as such, these transcription factors could provide attractive targets for innovative interventions to treat and prevent human breast cancers. Accordingly, our results demonstrate that reduction of NF-κB-dependent survival proteins (by simultaneous inhibition of IKK and CK2) synergizes with interferon-γ-mediated elevation of death signaling proteins to augment Apo2L/TRAIL-induced death of breast cancer cells.

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INTRODUCTION:

The clonal evolution of tumor cells in hypoxic microenvironments ultimately selects subpopulations that not only resist apoptosis, but also promote angiogenesis. The transcriptional regulators of the normal hypoxic response, Hypoxia Inducible Factor-1 (HIF-1) and NF-κB, are responsible for induction of genes that promote anaerobic metabolism, cell survival, vasodilatation, and angiogenesis. We hypothesize that cancer cells subvert these normal hypoxia-dependent mechanisms to enable their own deregulated survival, neovasculogenesis, and growth. We propose that inhibition of HIF-1 and/or NF-κB can abrogate the angiogenic and apoptosis-resistant phenotype of breast tumors, thereby curtailing their growth and metastases. We aim to elucidate the molecular mechanisms by which the p53 tumor suppressor regulates HIF-1and NF-κB activity and examine the effect of inhibiting HIF-1 and/or NF-κB on the growth, neovascularization, and metastatic potential of breast cancers *in vitro* and *in vivo*. These studies will provide insights into the molecular mechanisms governing the response to hypoxic stress and will determine whether their subversion by breast cancers is responsible for their apoptosis-resistant and angiogenic phenotype. These key transcription factors could provide targets for innovative interventions for the treatment and prevention of breast cancer.

BODY:

09/01/99 – 08/31/00:

The first annual report (*September 2000*) covered the first year (0-12 months) of the research project and was devoted to the successful completion of Specific Aim 1 (Tasks 1 and 2 of the statement of work).

Specific Aim 1. Investigate the mechanism(s) of p53-mediated repression HIF-1 and its role in regulation of hypoxia-induced angiogenesis.

- A. Elucidate the molecular mechanism(s) responsible for p53-mediated repression of HIF-1 activity.
- B. Define the role of HIF-1 in the angiogenic phenotype conferred by p53-deficiency

Statement of Work (1-12 months)

- Task 1: Elucidate the molecular mechanism(s) responsible for p53-mediated repression of HIF-1 activity.
- Task 2: Define the role of HIF-1 in the angiogenic phenotype conferred by p53-deficiency

We completed the studies proposed in specific aim 1 (Tasks 1, 2a,b) and published the results and conclusions in:

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Genes & Development* 14:34-44, 2000. (*Reprint of publication enclosed-Appendix 1*).

Abstract: The switch to an angiogenic phenotype is a fundamental determinant of neoplastic growth and tumor progression. We demonstrate that homozygous deletion of the p53 tumor suppressor gene via homologous recombination in a human cancer cell line promotes the neovascularization and growth of tumor xenografts in nude mice. We find that p53 promotes Mdm2-mediated ubiquitination and proteasomal degradation of the HIF-1α subunit of hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor that regulates cellular energy metabolism and angiogenesis in response to oxygen deprivation. Loss of p53 in tumor cells enhances HIF-1α levels and augments HIF-1-dependent transcriptional activation of the vascular endothelial growth factor (VEGF) gene in response to hypoxia. Forced expression of HIF-1α in p53-expressing tumor cells increases hypoxia-induced VEGF expression and augments neovascularization and growth of tumor xenografts. These results indicate that amplification of normal HIF-1-dependent responses to hypoxia via loss of p53 function contributes to the angiogenic switch during tumorigenesis.

09/01/00 – 08/31/01:

The previous report (*September 2001*) covered the period 12-24 months of the research project, and was devoted to Specific Aim 2 and part of Specific Aim 3 (Tasks 2 and 3 of the statement of work).

Specific Aim 2. Define the role of NF-κB RelA in the angiogenic phenotype conferred by p53 deficiency and the molecular determinants of κB-dependent angiogenesis

- A. Investigate whether repression of RelA by a transdominant mutant IκBα (IκBαM) can inhibit the angiogenic phenotype conferred by p53- deficiency.
- B. Investigate the molecular determinants of NF-κB-mediated angiogenesis.

Specific Aim 3. Examine the effect of inhibiting HIF-1 or RelA on growth, neovascularization, and metastatic potential of breast cancers.

Statement of Work (12-24 months):

Task 2: Define the role of NF-κB in the angiogenic phenotype conferred by p53-deficiency
Task 3: Define the role of NF-κB on growth and neovascularization of breast cancers.

We completed the studies proposed in specific aim 2 (Task 2) and part of specific aim 3 (Task 3a) and presented the results and conclusions in the following publication:

Ravi, R., Bedi, G.C., Engstrom, L., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J., & Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB. *Nature Cell Biology* **3**:409-416, (2001). (*Reprint of publication enclosed-Appendix 2*)

Abstract: While NF-κB promotes expression of death receptors (TRAIL-R1/R2, CD95/Fas), we have demonstrated that HER-2/neu-mediated activation of NF-κB (RelA) also induces expression of Bcl-x_L which protects breast cancer cells from Apo2L/TRAIL. Activation of NF-κB requires phosphorylation and ubiquitin-mediated degradation of IκBα via the activity of the IκB-kinase (IKK) complex containing two kinases (IKK-α and IKK-β) and the regulatory protein NEMO (NF-κB essential modifier; IKK-γ). A cell permeable peptide [NEMO binding domain (NBD) peptide] that blocks the interaction of NEMO with the IKK complex inhibits cytokine-induced NF-κB activation. Acetyl salicyclic acid (aspirin; ASA), also specifically inhibits the activity of IKK-β. Inhibition of NF-κB by blocking activation of the IκB-kinase complex with either a peptide that disrupts the interaction of IKKβ with NEMO or by acetyl salicylic acid (aspirin;ASA) reduces expression of Bcl-x_L and sensitizes breast cancer cells to Apo2L/TRAIL-induced death. The efficacy of Apo2L/TRAIL in the treatment of breast cancers may be improved by antibody-mediated inhibition of growth factor receptors (HER2/neu or IGF-1R) and/or peptidomimetic drugs that disrupt the IKK-NEMO complex.

Ravi, R. and Bedi, A. Sensitization of breast cancer cells to hypoxia-induced apoptosis by inhibition of NF-κB. (*Unpublished Data- Please refer to Appendix 3*).

Abstract: Electrophoretic mobility shift assays demonstrated that hypoxia induces NF-κB DNA-binding activity in 3T3 fibroblasts (Figure 1a). To examine the role of NF-κB in hypoxia-induced expression of VEGF, $RelA^{+/+}$ and $RelA^{-/-}$ 3T3 fibroblasts were analyzed for expression of HIF-1α protein and VEGF mRNA under tissue culture conditions simulating the hypoxic tumor microenvironment. Following exposure to 0.1% O_2, $RelA^{+/+}$ and $RelA^{-/-}$ cells exhibited equivalent induction of HIF-1α protein and VEGF mRNA (Figure 1b and c). However, expression of the anti-apoptotic Bcl-2 family member, Bcl-x_L, was markedly reduced in $RelA^{-/-}$ cells compared to their $RelA^{+/+}$ counterparts (Figure 1e). Although RelA was not required for hypoxia-induced expression of VEGF, $RelA^{-/-}$ cells exhibited greater levels of hypoxia-induced apoptosis than their $RelA^{+/+}$ counterparts (Figure 1d). Activation of NF-κB requires phosphorylation and degradation of IκBα via the activity of the IκB-kinase (IKK) complex. Inhibition of the IKK complex by the non-steroidal anti-inflammatory drug, aspirin (ASA) reduced hypoxia-induced expression of Bcl-x_L and sensitized cells to hypoxia-induced apoptosis (Figure 1d and e). Together, these results indicate that NF-κB promotes Bcl-x_L expression and protects tumor cells from hypoxia-induced apoptosis.

<u>09/01/01 – 08/31/02</u>:

The current report (*September 2002*) covers the period 24-36 months of the research project, and was devoted to Specific Aim 3 (Task 3 of the statement of work).

We completed the following studies and presented the results in the following publication and manuscript:

Ravi, R. and Bedi, A. Sensitization of Tumor Cells to Apo2L/TRAIL-induced Apoptosis by Inhibition of Casein Kinase II (CK2). *Cancer Research 62*: 4180-4185, 2002. (*Appendix 4*)

Abstract: Tumor cell death can be triggered by engagement of specific death receptors with Apo2L/TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). Apo2L/TRAIL-induced apoptosis involves caspase-8-mediated cleavage of BID. The active truncated form of BID (tBID) triggers the mitochondrial activation of caspase-9 by inducing the activation of BAK or BAX. Although breast cancer cell lines express death receptors for Apo2L/TRAIL, many remain resistant to TRAIL/Apo2L-induced death. Breast cancers frequently exhibit increased activity of casein kinase II (CK2). Here we demonstrate that CK2 is at the nexus of two signaling pathways that protect tumor cells from Apo2L/TRAIL-induced apoptosis. We find that CK2 inhibits Apo2L/TRAIL-induced caspase-8-mediated cleavage of BID, thereby reducing the formation of tBID. In addition, CK2 promotes NF-κB-mediated expression of Bcl-x_L, which sequesters tBID and curtails its ability to activate BAX. Tumor cells with

constitutive activation of CK2 exhibit a high Bcl-x_L/tBID ratio and fail to activate caspase-9 or undergo apoptosis in response to Apo2L/TRAIL. Conversely, reduction of the Bcl-x_L/tBID ratio by inhibition of CK2 renders such cancer cells sensitive to Apo2L/TRAIL-induced activation of caspase-9 and apoptosis. Using isogenic cancer cell lines that differ only in the presence or absence of either the *p53* tumor suppressor or the *BAX* gene, we show that the enhancement of Apo2L/TRAIL-induced tumor cell death by CK2 inhibitors requires BAX, but not p53. The identification of CK2 as a key survival signal that protects tumor cells from death receptor-induced apoptosis could aid the design of Apo2L/TRAIL-based combination regimens for treatment of diverse cancers.

Sensitization of breast cancer cells to death receptor-induced apoptosis by inhibition of NF-κB: Synergistic action of Apo2L/TRAIL, Interferon-γ, Aspirin and Apigenin. (Abstract presented at Era of Hope, 2002; Manuscript in preparation)(*Appendix 5*).

Abstract: Although Apo2L/TRAIL is a promising anticancer agent, several breast cancer cell lines remain resistant to Apo2L/TRAIL even though they express death receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5. Our findings demonstrate that cancer cells can be sensitized to Apo2L/TRAIL-induced death by interferon-γ-mediated upregulation of BAK, caspase-8, and caspase-7. The elevation of procaspase-8 potentiates Apo2L/TRAIL-mediated formation of tBID, which then interacts with the more abundant BAK to implement mitochondrial outer membrane permeabilization (MOMP) and caspase-9 activation even in the absence of BAX. Interferon-γ also facilitates caspase-9-mediated apoptotic signaling downstream of MOMP by increasing the amount of procaspase-7. While interferon-γ potentiates death receptor-induced apoptosis, Apo2L/TRAIL death signaling is counteracted by expression of NF-κB-inducible survival proteins, such as Bcl-x_L and IAPs (cIAP-2 and XIAP). Many breast cancers exhibit constitutively high NF-κB activity resulting from phosphorylation of IκB by IκB kinase (IKK) and/or casein kinase II (CK2). Our findings demonstrate that simultaneous inhibition of IKKβ (with acetyl salicylic acid, ASA), and CK2 (with the plant flavonoid, apigenin), results in loss of NF-κB-dependent expression of Bcl-x_L and IAPs, thereby potentiating activation of caspases-9 and -7, and promoting tumor cell apoptosis in response to Apo2L/TRAIL. We also show that the reduction of NF-κB-induced survival proteins by ASA and apigenin synergizes with interferon-γ–mediated elevation of death signaling proteins to augment Apo2L/TRAIL-induced apoptosis of breast cancer cells. Further studies are required to evaluate and optimize the therapeutic ratio of the combinatorial regimen of Apo2L/TRAIL, interferon-γ, aspirin, and apigenin for treatment of breast cancers.

Research Accomplished (*Period 24-36 months*):

Sensitization of Tumor Cells to Apo2L/TRAIL-induced Apoptosis by Inhibition of Casein Kinase II. Ravi, R. and Bedi, A. *Cancer Research 62*: 4180-4185, 2002. (*Appendix 4*)

Sensitization of breast cancer cells to death receptor-induced apoptosis by inhibition of NF-κB: Synergistic action of Apo2L/TRAIL, Interferon-γ, Aspirin and Apigenin. Ravi, R. and Bedi, A. Abstract presented at Era of Hope, 2002 (Manuscript in preparation) (*Appendix 5*).

Introduction

Genetic aberrations that render cells incapable of executing apoptosis underlie the observed resistance of human breast cancers to anticancer agents. Unraveling mechanisms to unleash the apoptotic program in tumor cells could provide effective therapeutic interventions against breast cancers.

Tumor cell death can be triggered by engagement of specific death receptors belonging to the tumor necrosis factor receptor gene superfamily with the "death ligand", Apo2L/TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). Apo2L/TRAIL-induced cell death involves caspase-8-mediated cleavage of BID to form truncated BID (tBID). tBID induces activation of BAX or BAK leading to mitochondrial outer membrane permeabilization (MOMP) and release of a cocktail of pro-death cofactors (such as cytochrome c, Smac/DIABLO) into the cytoplasm. The interaction of cytochrome c with Apaf-1 results in a nucleotide-dependent conformational change that allows binding and transactivation of caspase-9, which in turn, activates downstream caspases such as caspase-3 and caspase-7. The activation of caspases 9, 3, and 7, is further potentiated by Smac/DIABLO (second mitochondria-derived activator of caspase), a protein which binds and antagonizes the IAPs.

Apo2L/TRAIL induces apoptosis of many cancer cell lines *in vitro*, and its tumoricidal activity and safety *in vivo* has been confirmed in preclinical animal models of human breast cancer xenografts. However, many breast cancer cell lines express death receptors for Apo2L/TRAIL, yet remain relatively resistant to Apo2L/TRAIL-

induced apoptosis. The identification of the molecular determinants of Apo2L/TRAIL-induced death and key survival proteins that interrupt death receptor-induced signaling in tumor cells could aid the design of Apo2L/TRAIL-based combination regimens against breast cancers.

Results

1. *Tumor cell resistance to Apo2L/TRAIL-mediated apoptosis via loss of BAX, but not p53.*

HCT116 cells have wild type *p53* ($p53^{+/+}$) and an intact *BAX* allele ($BAX^{+/-}$), and express functional p53 and BAX proteins. Isogenic p53-deficient ($p53^{-/-}$) or BAX-deficient ($BAX^{-/-}$) derivatives of HCT116 cells were generated by targeted inactivation of either both *p53* alleles or the wild-type *BAX* allele in a BAX heterozygote (8, 9). Exposure of both BAX-proficient ($p53^{-/-}$ and $p53^{+/+}$) and $BAX^{-/-}$ HCT116 cells to Apo2L/TRAIL resulted in activation of caspase-8 and caspase-8-mediated proteolysis of BID (Fig. 1, 2). The formation of truncated BID (tBID) by Apo2L/TRAIL triggered the mitochondrial activation of caspase-9, and resulted in cleavage of caspase-7 and PARP in BAX-proficient HCT116 cells ($p53^{+/+}$ or $p53^{-/-}$)(Fig. 1, Fig. 2). In contrast, isogenic $BAX^{-/-}$ HCT116 cells failed to activate caspase-9 or caspase-7, and were resistant to Apo2L/TRAIL-induced apoptosis (Fig. 1, 2, 4). Therefore, Apo2L/TRAIL-induced apoptosis of cancer cells is independent of *p53*, but requires BAX.

2. *Interferon-γ augments the Apo2L/TRAIL-induced death signaling pathway.*

We examined the effect of interferon-γ on expression of the molecular components of the Apo2L/TRAIL-induced death signaling pathway in $BAX^{+/-}$ or $BAX^{-/-}$ isogenic tumor cells. Immunoblot analyses demonstrated that treatment with interferon-γ increased expression of the zymogens, caspase-8 and caspase-7, in both $BAX^{+/-}$ and $BAX^{-/-}$cells, but did not change expression of caspase-9 (Fig. 2). Treatment with interferon-γ also increased expression of BAK, without altering levels of BAX (Fig. 2). Since interferon-γ augmented expression of sequential determinants of the Apo2L/TRAIL-induced death signaling pathway (caspase-8, BAK, and caspase-7), we investigated whether interferon-γ can overcome the resistance of $BAX^{-/-}$ tumor cells to Apo2L/TRAIL. Pre-incubation of either $BAX^{+/-}$ or $BAX^{-/-}$ tumor cells with interferon-γ for 16h (and continued exposure for 48h in the presence of Apo2L/TRAIL) promoted formation of tBID, activation of caspase-9 and caspase-7, efficient cleavage of PARP, and induction of tumor cell death in response to Apo2L/TRAIL (Fig. 2, 4).

3. *Inhibition of Apo2L/TRAIL-induced apoptosis of tumor cells by Bcl-x_L*

tBID triggers mitochondrial outer membrane permeabilization (MOMP) by inducing the allosteric activation of BAK or BAX. To investigate whether the induction of apoptosis by the combination of Apo2L/TRAIL and interferon-γ is hindered by Bcl-x_L, we introduced a retroviral vector encoding Bcl-x_L into BAX-proficient HCT116 cells [Bcl-x_L($BAX^{+/-}$)]. Although Apo2L/TRAIL (with or without interferon-γ) induced formation of tBID, it could not activate caspases-9 or -7, and failed to induce apoptosis in BAX-proficient tumor cells overexpressing exogenous Bcl-x_L [Bcl-x_L($BAX^{+/-}$)] (Fig. 2, 4). Therefore, the ability of tBID to activate BAX or BAK is curtailed via its sequestration by Bcl-x_L.

4. *Apo2L/TRAIL-induced apoptosis is augmented by inhibiting NF-κB-dependent expression of Bcl-x_L and IAPs with acetyl salicylic acid and apigenin.*

The human *bcl-x* promoter contains a κB DNA site (TTTACTGCCC; 298/+22) responsible for its Rel-dependent induction. In addition to Bcl-x_L, members of the inhibitor of apoptosis family [cIAP-2 and X-chromosome linked IAP (XIAP)] are also NF-κB-induced proteins which inhibit caspases (-9, -7, -3). Activation of NF-κB requires phosphorylation of the inhibitory proteins, the IκBs, by either the IκB kinase (IKK) complex or casein kinase II (CK2). The IKKβ catalytic subunit of IKK is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), such as acetyl salicylic acid (aspirin) or sulindac sulfide, whereas CK2 is inhibited by the plant flavone, apigenin (Fig. 3). The combination of ASA (1 mM) with apigenin (10 μM) resulted in a greater repression of NF-κB DNA-binding activity and endogenous Bcl-x_L and IAPs (cIAP-2 and XIAP) than either agent alone (Fig. 3). Consistent with the reduced levels of Bcl-x_L and IAPs, treatment with the combination of ASA with apigenin potentiated activation of caspases-9 and -7, and induction of tumor cell apoptosis in response to Apo2L/TRAIL (Fig. 2, 4).

5. *Sensitization of breast cancer cells to Apo2L/TRAIL-induced apoptosis by the synergistic effects of IFN-γ and NF-κB inhibitors (aspirin and apigenin)*

Our results indicate that interferon-γ enhances expression of members of the Apo2L/TRAIL-death signaling pathway (caspase-8, BAK, and caspase-7), while the expression of NF-κB-induced survival proteins (Bcl-x_L and IAPs) is reduced by the combination of ASA and apigenin (Fig 2, 3). To investigate whether interferon-

γ–mediated elevation of death signaling proteins can synergize with the reduction of NF-κB-induced survival proteins to augment Apo2L/TRAIL-induced apoptosis, we examined the effect of a combinatorial regimen of interferon-γ, ASA, and apigenin, on the sensitivity of human breast cancer cell lines (MCF-7, SKBr-3, Hs578) to Apo2L/TRAIL-induced death. All three cell lines were sensitized to Apo2L/TRAIL-induced apoptosis by the synergistic effects of interferon-γ and NF-κB inhibitors (ASA, and apigenin)(Fig. 4).

Discussion:

Human breast cancer cell lines exhibit a wide heterogeneity in their sensitivity to TRAIL/Apo2L *in vitro*, and many remain resistant to Apo2L/TRAIL-induced apoptosis. These data suggest that successful treatment of breast cancers with TRAIL/Apo2L may require its combination with agents that inhibit survival signals responsible for protecting tumor cells from death receptor-induced apoptosis.

Apo2L/TRAIL-induced cell death involves caspase-8-mediated cleavage of BID to form truncated BID (tBID). tBID induces activation of BAX or BAK leading to mitochondrial outer membrane permeabilization (MOMP) and release of a cocktail of pro-death cofactors (such as cytochrome c, Smac/DIABLO) into the cytoplasm. Since many breast cancer cells exhibit decreased expression of BAX (*unpublished observations*), our data suggest that BAX-deficiency may render breast cancer cells resistant to Apo2L/TRAIL-induced apoptosis. In addition, amplification and consequent overexpression c-erbB2 (HER-2/neu) or IGF-1 receptor (IGF-1R) is observed in a significant proportion of human breast cancers. Both HER-2/neu and IGF-1R promote PI3 kinase (PI3-K)-mediated phosphorylation and activation of Akt, a serine-threonine kinase that, in turn, activates the IκB kinase (IKK) complex. The activated IKK complex induces phosphorylation-mediated degradation of IκB, thereby promoting activation of NF-κB. In addition to aberrant activation of the IKK complex, breast cancers frequently exhibit increased activity of casein kinase II (CK2). The activation of either IKK or CK2 results in constitutive NF-κB activity in breast cancer cells. Our results indicate that NF-κB protects breast cancer cells from Apo2L/TRAIL-induced apoptosis by promoting expression of Bcl-x_L, a Bcl-2 family member that sequesters tBID and inhibits activation of BAX. In addition to Bcl-x_L, NF-κB also protects tumor cells from Apo2L/TRAIL-induced apoptosis by inducing expression of members of the inhibitor of apoptosis family [cIAP-2 and X-chromosome linked IAP (XIAP)].

Our findings demonstrate that cancer cells can be sensitized to Apo2L/TRAIL-induced death by interferon-γ-mediated upregulation of BAK, caspase-8, and caspase-7. The elevation of procaspase-8 potentiates Apo2L/TRAIL-mediated formation of tBID, which then interacts with the more abundant BAK to implement mitochondrial outer membrane permeabilization (MOMP) and caspase-9 activation even in the absence of BAX. Interferon-γ also facilitates caspase-9-mediated apoptotic signaling downstream of MOMP by increasing the amount of procaspase-7. While interferon-γ potentiates death receptor-induced apoptosis, Apo2L/TRAIL death signaling is counteracted by expression of NF-κB-inducible survival proteins, such as Bcl-x_L and IAPs (cIAP-2 and XIAP). Our findings demonstrate that simultaneous inhibition of IKKβ (with acetyl salicylic acid, ASA), and CK2 (with the plant flavonoid, apigenin), results in loss of NF-κB-dependent expression of Bcl-x_L and IAPs, thereby potentiating activation of caspases-9 and -7, and promoting tumor cell apoptosis in response to Apo2L/TRAIL. We also show that the reduction of NF-κB-induced survival proteins by ASA and apigenin synergizes with interferon-γ–mediated elevation of death signaling proteins to augment Apo2L/TRAIL-induced apoptosis of breast cancer cells.

Our results suggest that the following Apo2L/TRAIL-based combination regimens may be useful for the treatment of human breast cancers:

1. Apo2L/TRAIL + Interferon-γ + inhibitors of growth factor receptor tyrosine kinases (HER-2/neu) (trastuzumab).
2. Apo2L/TRAIL + interferon-γ + aspirin + apigenin
3. Apo2L/TRAIL + interferon-g + NF-κB inhibitors (PS-341 or parthenolide)

Further studies are required to evaluate and optimize the therapeutic ratio of these combinatorial regimens.

KEY RESEARCH ACCOMPLISHMENTS:

- Our observations indicate that loss of p53 function, via somatic mutations or expression of viral oncoproteins, contributes to activation of the angiogenic switch and promotes tumor growth.

- Our studies define a novel mechanism by which p53 regulates the angiogenic switch; p53 inhibits hypoxia-induced expression of HIF-1α by facilitating its ubiquitination and subsequent degradation.
- Our findings suggest that amplification of HIF-1 activity resulting from loss of p53 function may contribute to the overexpression of VEGF that is observed in a wide variety of human cancers.
- Our results indicate that NF-κB/RelA is required for hypoxia-induced expression of Bcl-x_L and protection of cells from hypoxia- and death receptor-induced apoptosis.
- Our findings indicate that activation of NF-κB by HER-2/neu or insulin-like growth factor-1 (IGF-1) renders breast cancer cells relatively resistant to Apo2L/TRAIL-induced apoptosis. Conversely, breast cancer cells can be sensitized to Apo2L/TRAIL-induced death by antibody-mediated inhibition of growth factor receptors (HER2/neu or IGF-1R) and/or inhibitors of the IKK complex.
- Our findings have identified casein kinase-II (CK2) as a key survival signal that activates NF-κB and protects tumor cells from Apo2L/TRAIL-induced apoptosis. Conversely, breast cancer cells can be sensitized to Apo2L/TRAIL-induced death by inhibition of CK2 with the plant flavone, apigenin.
- Our findings demonstrate that breast cancer cells can be sensitized to Apo2L/TRAIL-induced death by interferon-γ-mediated upregulation of death signaling proteins (caspase-8, BAK, and caspase-7). Interferon-γ–mediated elevation of death signaling proteins synergizes with the reduction of NF-κB-induced survival proteins by aspirin and apigenin to augment Apo2L/TRAIL-induced apoptosis of breast cancer cells. The combination of Apo2L/TRAIL, interferon-γ, aspirin, and apigenin may be an effective regimen for treatment of breast cancers.

REPORTABLE OUTCOMES:

Manuscripts/ Abstracts/ Presentations:

We have completed the studies proposed in specific aims 1, 2, and 3 (Tasks 1,2, and 3) and have reported the results and conclusions in:

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Genes & Development* 14:34-44, 2000. (*Appendix 1-Reprint of publication enclosed*).

These findings were presented (abstract & poster) at the AACR-NCI-EORTC Meeting in Washington, D.C., 1999.

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Abstract & Presentation at AACR-NCI-EORTC Meeting*, Washington, D.C., Nov. 1999.

Ravi, R., Bedi, G.C., Engstrom, L., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J., & Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB. *Nature Cell Biology* 3:409-416, (2001). (*Appendix 2-Reprint of publication enclosed*)

Ravi, R. and Bedi, A. Sensitization of Tumor Cells to Apo2L/TRAIL-induced Apoptosis by Inhibition of Casein Kinase II. *Cancer Research 62*: 4180-4185, 2002. (*Appendix 4*)

Ravi, R., Prouser, T and Bedi, A. Sensitization of breast cancer cells to death receptor-induced apoptosis by inhibition of NF-κB: Synergistic action of Apo2L/TRAIL, Interferon-γ, Aspirin and Apigenin. *Abstract presented at Era of Hope, Orlando, FL, September 2002 (Manuscript in preparation)* (*Appendix 5*).

Ravi, R and Bedi, A. Role of Death Receptors in Apoptosis, *Genetics of Apoptosis*. BIOS Scientific Publishers, Oxford, U.K.. Editor – Grimm, S., 2002 (In Press).(*Appendix 6*)

CONCLUSIONS:

Importance of completed research:

There are two major impediments to the successful treatment of breast cancer. First, surgical extirpation of the primary neoplasm is often followed by the occurrence of metastatic tumors. Second, overt metastases are

resistant to conventional chemo- or radio-therapy. Therefore, successful treatment is contingent upon identifying strategies to prevent metastases or eliminate tumor cells that have acquired genetic aberrations that confer resistance to cytotoxic agents. Our results indicate that amplification of HIF-1 activity resulting from loss of p53 function may contribute to the angiogenic phenotype of human cancers. Conversely, inhibition of HIF-1 may provide a therapeutic strategy to curtail the tumor growth and progression. We have also determined that activation of NF-κB promotes expression of Bcl-x_L protects cells from hypoxia-induced apoptosis. Our findings provide a scientific foundation for targeting HIF-1 and NF-κB to overcome the hypoxia-resistant angiogenic phenotype of breast cancers.

Apo2L/TRAIL induces apoptosis of many cancer cell lines *in vitro*, and its tumoricidal activity and safety *in vivo* has been confirmed in preclinical animal models of human breast cancer xenografts. However, many breast cancer cell lines express death receptors for Apo2L/TRAIL, yet remain relatively resistant to Apo2L/TRAIL-induced apoptosis. Our data suggest that breast cancer cells may be rendered resistant to Apo2L/TRAIL-induced apoptosis by deficiency of BAX, a pro-apoptotic member of the Bcl-2 family. Our findings demonstrate that BAX-deficient cancer cells can be sensitized to Apo2L/TRAIL-induced death by interferon-γ-mediated upregulation of BAK, caspase-8, and caspase-7.

While interferon-γ potentiates death receptor-induced apoptosis, Apo2L/TRAIL death signaling is counteracted by expression of NF-κB-inducible survival proteins, such as Bcl-x_L and IAPs (cIAP-2 and XIAP). Amplification and consequent overexpression c-erbB2 (HER-2/neu) or IGF-1 receptor (IGF-1R) is observed in a significant proportion of human breast cancers. Both HER-2/neu and IGF-1R promote PI3 kinase (PI3-K)-mediated phosphorylation and activation of Akt, a serine-threonine kinase that, in turn, activates the IκB kinase (IKK) complex. The activated IKK complex induces phosphorylation-mediated degradation of IκB, thereby promoting activation of NF-κB. In addition to aberrant activation of the IKK complex, breast cancers frequently exhibit increased activity of casein kinase II (CK2). Many breast cancers exhibit constitutively high NF-κB activity resulting from phosphorylation of IκB by IκB kinase (IKK) and/or casein kinase II (CK2). Our findings demonstrate that simultaneous inhibition of IKKβ (with acetyl salicylic acid, ASA), and CK2 (with the plant flavonoid, apigenin), results in loss of NF-κB-dependent expression of Bcl-x_L and IAPs, thereby promoting tumor cell apoptosis in response to Apo2L/TRAIL. We also show that the reduction of NF-κB-induced survival proteins by ASA and apigenin synergizes with interferon-γ–mediated elevation of death signaling proteins to augment Apo2L/TRAIL-induced apoptosis of breast cancer cells. The identification of the molecular determinants of Apo2L/TRAIL-induced death and key survival proteins that interrupt death receptor-induced signaling in tumor cells could aid the design of Apo2L/TRAIL-based combination regimens against breast cancers.

Implications and practical applications of completed research:

Strategies to inhibit angiogenesis have hitherto focused upon inhibition of individual angiogenic factors/receptors or suppression of endothelial cell proliferation. Unlike these approaches which target downstream mediators of angiogenesis, strategies that target the proximal transcriptional mediators of angiogenesis and cell survival (HIF-1 or NF-κB) would be expected to inhibit an entire panel of synergizing factors. As such, it may be more potent and less susceptible to evasion by genetically pliable tumor cells that could evolve mechanisms of resistance against any individual factor. By demonstrating that deregulation of HIF-1 contributes to the increased expression of VEGF in p53-deficient cancers, our data provide further support for the hypothesis that inhibition of HIF-1 may abrogate the ability of such tumors to establish an adequate vascular supply and adapt their cellular metabolism to hypoxia, thereby curtailing their growth and metastasis. By identifying NF-κB as a key determinant of tumor cell survival, our studies suggest that inhibition of NF-κB by drugs that target the IKK complex and inhibit casein kinase II may be used to potentiate Apo2L/TRAIL-induced death of breast cancer cells.

Our results suggest that the following Apo2L/TRAIL-based combination regimens may be useful for the treatment of human breast cancers:

1. Apo2L/TRAIL + Interferon-γ + inhibitors of growth factor receptor tyrosine kinases (HER-2/neu) (trastuzumab).
2. Apo2L/TRAIL + interferon-γ + aspirin + apigenin
3. Apo2L/TRAIL + interferon-γ + NF-κB inhibitors (PS-341 or parthenolide)

Future Studies:

We have initiated studies to evaluate and optimize the therapeutic ratio of the Apo2L/TRAIL-based combinatorial regimens described above.

REFERENCES:

The references pertinent to the report are listed in the appended publications and manuscript (Appendices 1,2,4,6).

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Genes & Development* 14:34-44, 2000. (*Appendix 1-Reprint of publication enclosed*).

Ravi, R., Bedi, G.C., Engstrom, L., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J., & Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB. *Nature Cell Biology* **3**:409-416, (2001). (*Appendix 2-Reprint of publication enclosed*)

Ravi, R. and Bedi, A. Sensitization of Tumor Cells to Apo2L/TRAIL-induced Apoptosis by Inhibition of Casein Kinase II. *Cancer Research 62*: 4180-4185, 2002. (*Appendix 4-Reprint of publication enclosed*)

Ravi, R and Bedi, A. Role of Death Receptors in Apoptosis, *Genetics of Apoptosis*. BIOS Scientific Publishers, Oxford, U.K.. Editor – Grimm, S., 2002 (In Press).(*Appendix 6-Reprint of publication enclosed*)

APPENDICES:

Appendix 1:

Reprint of publication: (Page Numbers 13-23)

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. Genes & Development 14:34-44, 2000.

Appendix 2:

Reprint of publication: (Page Numbers 24-32)

Ravi, R., Bedi, G.C., Engstrom, L., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J., & Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB. *Nature Cell Biol*ogy **3**:409-416, (2001).

Appendix 3:

Unpublished Data: (Page 33-34)

Figure 1

Ravi, R. and Bedi, A. Sensitization of breast cancer cells to hypoxia-induced apoptosis by inhibition of NF-κB.

Appendix 4:

Reprint of publication: (Page Numbers 35-40)

Ravi, R. and Bedi, A. Sensitization of Tumor Cells to Apo2L/TRAIL-induced Apoptosis by Inhibition of Casein Kinase II. *Cancer Research 62*: 4180-4185, 2002.

Appendix 5:

Unpublished Data: (Page 41-51)

Figures 1-4

Ravi, R., Prouser, T and Bedi, A. Sensitization of breast cancer cells to death receptor-induced apoptosis by inhibition of NF-κB: Synergistic action of Apo2L/TRAIL, Interferon-γ, Aspirin and Apigenin.

Abstract presented at Era of Hope, Orlando, FL, September 2002 (Manuscript in preparation)

Appendix 6:

Reprint of publication: (Page Numbers 52-96)

Ravi, R and Bedi, A. Role of Death Receptors in Apoptosis, *Genetics of Apoptosis*. BIOS Scientific Publishers, Oxford, U.K.. Editor – Grimm, S., 2002 (In Press)

Appendix 1: Reprint of publication: (Page Numbers 13-23)

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., Bedi, A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. Genes & Development 14:34-44, 2000.

Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α

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The switch to an angiogenic phenotype is a fundamental determinant of neoplastic growth and tumor progression. We demonstrate that homozygous deletion of the *p53* tumor suppressor gene via homologous recombination in a human cancer cell line promotes the neovascularization and growth of tumor xenografts in nude mice. We find that p53 promotes Mdm2-mediated ubiquitination and proteasomal degradation of the HIF-1α subunit of hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor that regulates cellular energy metabolism and angiogenesis in response to oxygen deprivation. Loss of *p53* in tumor cells enhances HIF-1α levels and augments HIF-1-dependent transcriptional activation of the vascular endothelial growth factor (*VEGF*) gene in response to hypoxia. Forced expression of HIF-1α in p53-expressing tumor cells increases hypoxia-induced *VEGF* expression and augments neovascularization and growth of tumor xenografts. These results indicate that amplification of normal HIF-1-dependent responses to hypoxia via loss of p53 function contributes to the angiogenic switch during tumorigenesis.

[*Key Words*: p53; hypoxia-inducible factor-1 (HIF-1); angiogenesis; vascular endothelial growth factor (VEGF); hypoxia; cancer]

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Regions of vascular deficiency or defective microcirculation in growing tumors are deprived of O_2, glucose, and other nutrients. Apoptosis induced by nutrient deficiency counterbalances cell proliferation and limits tumor growth (Holmgren et al. 1995; O'Reilly et al. 1996; Parangi et al. 1996). Clonal evolution of tumor cells in this hypoxic microenvironment results from selection of subpopulations that not only resist apoptosis (Graeber et al. 1996) but also promote the formation of new blood vessels (for review, see Hanahan and Folkman 1996; Folkman 1997). In addition to promoting further growth of the primary tumor, cellular adaptation to hypoxia and tumor neovascularization strongly correlate with the risk of invasion and metastasis (Brown and Giaccia 1998; Dang and Semenza 1999; for review, see Folkman 1997). The switch to an angiogenic phenotype is considered to be a fundamental determinant of neoplastic progression (Gimbrone et al. 1972; Folkman et al. 1989; Bergers et al. 1999). This realization has, in turn, fueled an intense search for the molecular mechanisms by which the angiogenic switch is activated during tumorigenesis.

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Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that regulates O_2 homeostasis and physiologic responses to O_2 deprivation (for review, see Guillemin and Krasnow 1997; Semenza 1999). HIF-1 consists of two subunits, HIF-1α and HIF-1β, that belong to a subfamily of basic helix-loop-helix (bHLH) transcription factors containing a PAS (Per-ARNT-Sim) motif (Wang et al. 1995). A decrease in cellular O_2 tension leads to elevation of HIF-1 activity via stabilization of the HIF-1α protein; conversely, ubiquitin-mediated proteolysis of HIF-1α on reexposure to a normoxic environment results in rapid decay of HIF-1 activity (Semenza and Wang 1992; Wang et al. 1995; Salceda and Caro 1997; Huang et al. 1998; Kallio et al. 1999). The binding of HIF-1α, in conjunction with its dimerization partner HIF-1β, to DNA (consensus binding sequence, 5′-RCGTG-3′) leads to the transcriptional activation of genes that mediate anaerobic metabolism (glucose transporters and glycolytic enzymes), O_2-carrying capacity (erythropoietin, transferrin), and vasodilatation (inducible nitric oxide synthase and heme oxygenase-1) (for review, see Guillemin and Krasnow 1997; Semenza 1999). HIF-1 also binds to the 5′ flanking sequence of the vascular endothelial growth factor (*VEGF*) gene and is required for transactivation of *VEGF* in response to hypoxia (Forsythe

et al. 1996; Carmeliet et al. 1998; Iyer et al. 1998; Ryan et al. 1998). The binding of VEGF to the receptor tyrosine kinases flk1/KDR, flt-1, and flt-4 (VEGFR-1–VEGFR-3) on vascular endothelial cells promotes their proliferation and leads to vessel formation (for review, see Ferrara 1993; Risau and Flamme 1995; Brown et al. 1996). In contrast to wild-type cells, *VEGF* gene expression is not induced by hypoxia in HIF-1α-deficient embryonic stem cells, and dramatic vascular regression occurs in HIF-1α-null mouse embryos (Iyer et al. 1998; Kotch et al. 1999). Therefore, HIF-1 is a key transcriptional mediator of metabolic adaptation and VEGF-mediated angiogenesis in response to hypoxia. Although these responses serve to maintain O_2 homeostasis in normal tissues, they are also co-opted by tumors to facilitate neovascularization and growth. Akin to their role in vascular development and remodeling in normal tissues, HIF-1α (Maxwell et al. 1997; Carmeliet et al. 1998; Ryan et al. 1998) and VEGF (Plate et al. 1992; Shweiki et al. 1992; Kim et al. 1993; Millauer et al. 1994) facilitate tumor angiogenesis, and both HIF-1α (Zhong et al. 1999) and VEGF (for review, see Folkman 1997) are overexpressed in a wide variety of human cancers.

The genetic alterations that are responsible for oncogenesis and tumor progression may also underlie the ability of tumors to switch to an angiogenic phenotype. The human *p53* tumor suppressor gene encodes a multifunctional transcription factor that mediates cellular responses to diverse stimuli, including DNA damage and hypoxia (for review, see Giaccia and Kastan 1998). In addition to being an integral component of the surveillance mechanisms that arrest cell cycle progression under adverse conditions, p53 is also involved in mediating hypoxia-induced apoptosis (Graeber et al. 1996) and inducing inhibitors of angiogenesis such as thrombospondin-1 (Dameron et al. 1994; Van Meir et al. 1994). Evidence also suggests that p53 negatively regulates *VEGF* expression (Mukhopadhyay et al. 1995; Bouvet et al. 1998; Fontanini et al. 1998). Somatic mutations of the *p53* gene represent one of the most common genetic alterations in human cancers, and the acquisition of such defects is strongly associated with tumor progression and metastasis (for review, see Levine 1997).

In this study, we demonstrate that genetic inactivation of p53 in cancer cells provides a potent stimulus for tumor angiogenesis and identify a novel mechanism by which loss of*p53* function contributes to activation of the angiogenic switch in tumors. We find that homozygous deletion of p53 via homologous recombination in human colon cancer cells promotes the neovascularization and growth of tumor xenografts in nude mice. We show that p53 inhibits HIF-1 activity by targeting the HIF-1α subunit for Mdm2-mediated ubiquitination and proteasomal degradation. Conversely, the loss of p53 enhances hypoxia-induced HIF-1α levels and augments HIF-1-dependent expression of VEGF in tumor cells. We further demonstrate that forced expression of HIF-1α in p53-expressing tumor cells promotes *VEGF* expression and neovascularization of tumor xenografts. These findings indicate that inactivation of p53 in tumor cells contributes to activation of the angiogenic switch via amplification of normal HIF-1-dependent responses to hypoxia.

Results

Inhibition of tumor angiogenesis and growth by p53

The effect of p53 on tumor cell growth and angiogenesis was examined by comparing an isogenic set of human colon adenocarcinoma cell lines differing only in their p53 status (Bunz et al. 1998). The parental HCT116 line, containing wild-type p53 ($p53^{+/+}$), and a p53-deficient derivative ($p53^{-/-}$), generated by homologous recombination, demonstrated equivalent growth kinetics in tissue culture, with doubling times of 29 and 32 hr, respectively (Fig. 1A). However, xenografts (2.5×10^4–2.5×10^5 cells) of $p53^{-/-}$ HCT116 cells in athymic BALB/c (nu/nu) mice exhibited a significantly shorter latency and marked increase in tumor growth kinetics compared with their $p53^{+/+}$ counterparts (Fig. 1B,C). Whereas 12/12 animals inoculated with 2.5×10^4 $p53^{-/-}$ cells developed tumors within 3 weeks, only 1/12 mice receiving the same number of $p53^{+/+}$ cells was able to establish a tumor during the entire 8-week observation period. To examine whether the observed differences in growth kinetics in vivo were associated with variation in tumor vascularity, tumors established from $p53^{+/+}$ and $p53^{-/-}$ cells were subjected to histologic analysis and nuclear magnetic resonance (NMR) imaging. Immunohistochemical analyses of tumor sections using an antibody against von Willebrand Factor (vWF) demonstrated significantly increased blood vessel density in $p53^{-/-}$ tumors compared with their $p53^{+/+}$ counterparts (Fig. 1D,E). Analyses of neovascularization by NMR imaging showed that compared with $p53^{+/+}$ tumors, $p53^{-/-}$ tumors had a higher vascular volume (14 ± 2.6 μl/g vs. 8.4 ± 2.4 μl/g in highly permeable regions), as well as a threefold greater vascular permeability (0.4 ± 0.18 μl/g/min vs. 0.13 ± 0.04 μl/g/min in highly vascular zones) (Fig. 1F). Thus, loss of p53 function has a profound effect on the neovascularization and growth of human colorectal cancer xenografts in nude mice.

Effect of p53 genotype on hypoxia-induced VEGF *expression and HIF-1 activity*

Hypoxia-induced, HIF-1-mediated expression of VEGF stimulates angiogenesis and vascular permeability in neoplastic tissues (Plate et al. 1992; Shweiki et al. 1992; Forsythe et al. 1996; Maxwell et al. 1997; Carmeliet et al. 1998). $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells were analyzed for expression of VEGF mRNA and protein under tissue culture conditions simulating the hypoxic tumor microenvironment. Following exposure to 1% O_2, $p53^{-/-}$ cells exhibited a greater induction of VEGF mRNA and protein compared with their $p53^{+/+}$ counterparts (Fig. 2A,B). Transcriptional activation of the *VEGF* gene in response to hypoxia is mediated by binding of HIF-1 to a 47-bp

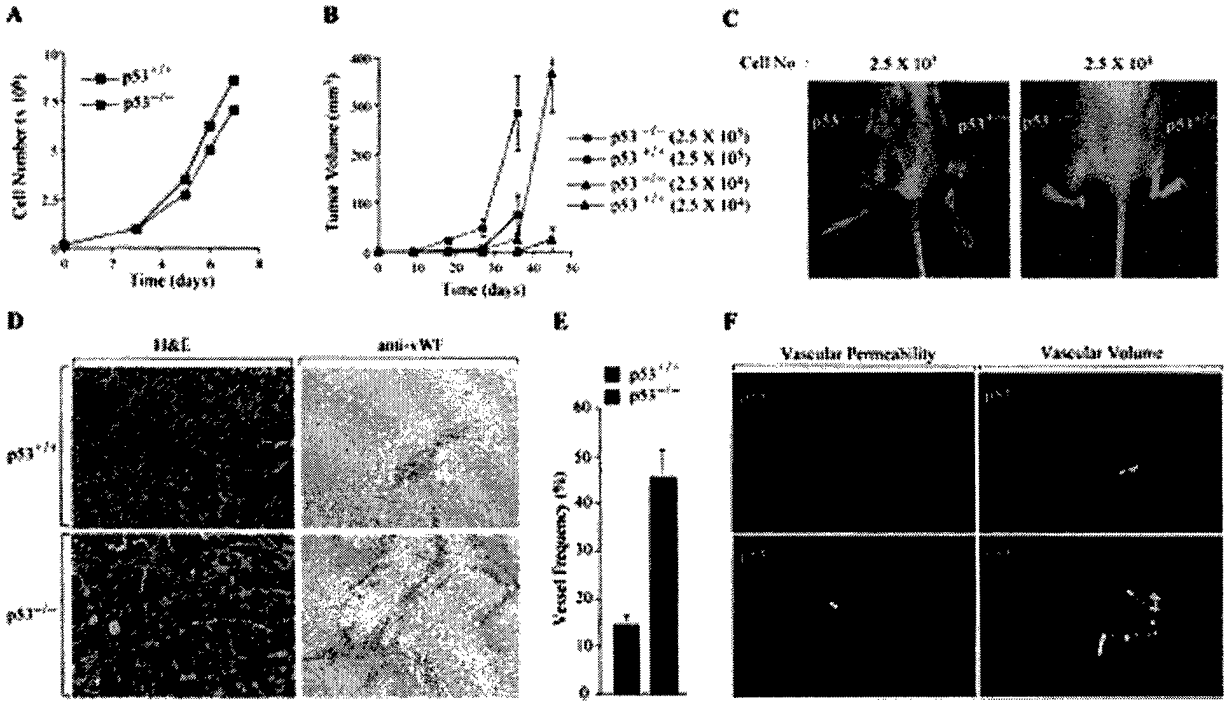

Figure 1. Effect of p53 genotype on tumor growth and angiogenesis. (*A*) Growth of $p53^{+/+}$ (blue) and $p53^{-/-}$ (red) HCT116 cells cultured in DMEM supplemented with 10% fetal calf serum at 37°C and 95%air/5%CO_2. (*B, C*) Growth of $p53^{+/+}$ (blue) and $p53^{-/-}$ (red) HCT116 xenografts [2.5×10^4 (▲) or 2.5×10^5 (■) cells] injected subcutaneously into right ($p53^{+/+}$) or left ($p53^{-/-}$) hind legs of athymic BALB/c (nu/nu) mice. Values expressed represent mean ± S.E. of 12 xenografts of each cell type. (*D*) Histologic analysis of blood vessels in $p53^{+/+}$ and $p53^{-/-}$ HCT116 xenograft tumors by staining with H&E or immunoperoxidase detection of endothelial cells using an anti-vWF antibody (×25). (*E*) Quantification of blood vessel density in $p53^{+/+}$ (blue) and $p53^{-/-}$ (red) xenografts. The data represent the mean ± S.E. of the frequency of vessel hits among 300 random sampling points from each of three tumors of either genotype. (*F*) Representative NMR analysis of in vivo vascular volume (*right*) and permeability (*left*) of $p53^{+/+}$ and $p53^{-/-}$ (*bottom*) HCT116 xenografts.

hypoxia-response element in the 5′ flanking region, and a reporter plasmid containing this sequence (VEGF-p11w) is transactivated by cotransfection of an expression vector encoding HIF-1α (pCEP4/HIF-1α) (Forsythe et al. 1996). To examine whether p53 influences HIF-1-mediated transcriptional activation of VEGF, $p53^{+/+}$ and $p53^{-/-}$ cells were cotransfected with the VEGF-p11w reporter and CMVβgal [encoding β-galactosidase (β-gal)]. Analyses of luciferase and β-gal activity in response to hypoxia (1% O_2) revealed a fourfold greater increase in VEGF-p11w transcription (relative to β-gal) in $p53^{-/-}$ cells compared with $p53^{+/+}$ cells (Fig. 2C). These differences were not seen when the reporter contained a 3-bp substitution in the hypoxia response element that eliminated HIF-1 binding (VEGF-p11m), suggesting that HIF-1 was a target for p53-mediated inhibition. Coexpression of pCEP4/HIF-1α in $p53^{+/+}$ cells increased hypoxia-induced activation of VEGF-p11w to levels that approached the reporter activity exhibited by hypoxic $p53^{-/-}$ cells in the absence of exogenous HIF-1α (Fig. 2C). Conversely, cotransfection of an expression vector encoding wild-type human p53 into $p53^{-/-}$ cells completely repressed hypoxia-induced VEGF-p11w expression (Fig. 2C). Electrophoretic mobility shift assays demonstrated that hypoxia-induced HIF-1 DNA-binding activity was reduced in $p53^{+/+}$ cells compared with $p53^{-/-}$ cells (Fig. 2D). The specificity of binding of HIF-1 to DNA was confirmed by competing hypoxia-induced DNA–protein complexes with excess unlabeled wild-type probe but not with an unlabeled mutant probe containing the same 3-bp substitution in the HIF-1 binding site as in reporter VEGF-p11m. Thus, p53 inhibits HIF-1 activity and *VEGF* expression in response to hypoxia.

Effect of p53 on oxygen-regulated expression and stability of HIF-1α

Hypoxia-induced HIF-1 DNA-binding and transcriptional activity are dependent on increased levels of HIF-1α protein and its heterodimerization with HIF-1β (Wang and Semenza 1993; Wang et al. 1995; Jiang et al. 1996; Huang et al. 1998). To investigate whether p53 influences HIF-1 activity by altering expression of HIF-1α, the levels of HIF-1α protein and mRNA were assessed in $p53^{+/+}$ and $p53^{-/-}$ cells exposed to either 20% or 1% O_2. In response to hypoxia, $p53^{-/-}$ HCT116 cells or mouse embryonic fibroblasts (MEFs) expressed higher levels of HIF-1α protein compared with their $p53^{+/+}$ counterparts (Fig. 3A,B). In contrast to HIF-1α protein levels, HIF-1α mRNA was expressed at equivalent levels

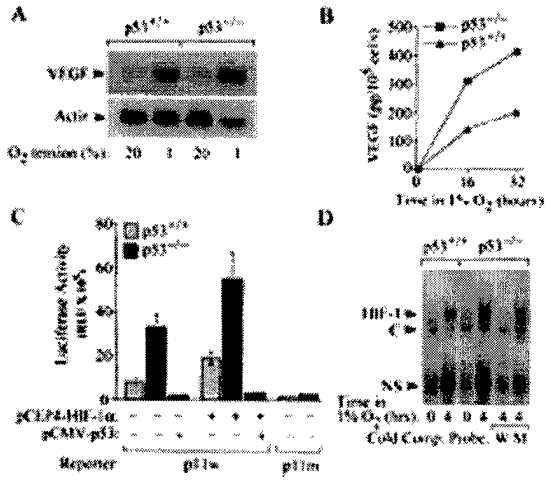

Figure 2. Effect of p53 genotype on hypoxia-induced *VEGF* expression and HIF-1 activity. (*A*) Northern blot analysis of VEGF mRNA expression in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells incubated for 16 hr in either 20%or 1%O_2. (*B*) ELISA of VEGF protein concentration in supernatant medium of $p53^{+/+}$ (blue ▲) or $p53^{-/-}$ (red ■) HCT116 cells incubated for 16–32 hr in 1% O_2. (*C*) Hypoxia-induced and HIF-1-dependent activation of VEGF-reporter activity in $p53^{+/+}$ (shaded bars) and $p53^{-/-}$ (solid bars) HCT116 cells. Wild-type (p11w) and mutant (p11m) copies of the hypoxia response element from the *VEGF* gene were inserted 5′ to a SV40 promoter–luciferase transcription unit. Cells were cotransfected with either VEGF–p11w or VEGF–p11m and CMVβgal, with or without pCEP4/HIF-1α or pCMV–p53, exposed to 1% O_2 for 20 hr, and harvested for luciferase assays. The data represent the mean ± S.E. luciferase activity (normalized for β-gal activity) from three independent experiments. (*D*) Electrophoretic mobility shift assays of HIF-1 DNA-binding activity in nuclear extracts from $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells exposed to 20% (lanes *1* and *3*) or 1% (lanes *2* and *4–6*) O_2. HIF-1 DNA binding was confirmed by competition assays using either unlabeled wild-type oligonucleotide (W) or a mutant oligonucleotide (M) containing the same 3-bp substitution as in p11m. Complexes containing HIF-1, constitutive (C), and nonspecific (NS) DNA-binding activities (Semenza and Wang 1992) are indicated.

in hypoxic $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 3C), suggesting an effect of p53 on HIF-1α protein expression. To confirm this effect, $p53^{-/-}$ cells were cotransfected with pCEP4-HIF-1α and either pCMV-p53 (encoding wild-type human p53) or empty vector (pCMV0) and exposed to 1% O_2 for 8 hr. Immunoblot analysis showed that $p53^{-/-}$ cells cotransfected with pCMV-p53 exhibited reduced levels of HIF-1α compared with cells receiving the control vector (Fig. 3D).

The steady state level of HIF-1α protein is regulated by an oxygen-dependent and iron-sensitive mechanism of ubiquitin-mediated proteasomal degradation (Salceda and Caro 1997; Huang et al. 1998; Kallio et al. 1999). The 20S proteasome is the core catalytic subunit of the 26S proteasome complex that mediates degradation of ubiquitin-tagged proteins (for review, see Hershko and Ciechanover 1998). HIF-1α expression is induced by exposure to hypoxia or treatment with cobalt chloride (Wang et al. 1995). To examine whether p53 influences the stability of HIF-1α protein, HIF-1α expression was analyzed in lysates of cobalt-treated $p53^{+/+}$ and $p53^{-/-}$ cells at serial time intervals following addition of cycloheximide. HIF-1α protein decayed with a half-life of <20 min in $p53^{+/+}$ cells, compared with >40 min in $p53^{-/-}$ cells (Fig. 3E).

HPV-E6 augments HIF-1α stability and VEGF *expression in response to hypoxia*

The human papilloma virus (HPV16) E6 oncoprotein promotes ubiquitin-dependent conjugation and degradation of p53 (Scheffner et al. 1990). To investigate whether E6-induced degradation of endogenous p53 promotes expression of HIF-1α and induction of VEGF, the PA-1 ovarian teratocarcinoma cell line was stably transfected with an expression vector encoding HPV-16 E6 (PA-1 E6) or empty vector (PA-1 Neo) (Ravi et al. 1998). Under hypoxic conditions, PA-1 E6 cells expressed higher levels of HIF-1α protein compared with PA-1 Neo cells (Fig. 4A). Analyses of HIF-1α protein stability in cycloheximide-treated cells showed that HIF-1α protein decayed with a half-life of ~15 min in PA-1 cells, compared with >30 min in PA-1 E6 cells (Fig. 4B). PA-1 Neo or PA-1 E6 cells were cotransfected with either VEGF-p11w or VEGF-p11m reporter and CMVβgal. Analyses of luciferase and β-gal activity in response to hypoxia (1% O_2) revealed a twofold greater increase in VEGF-p11w transcription (relative to β-gal) in PA-1 E6 cells compared

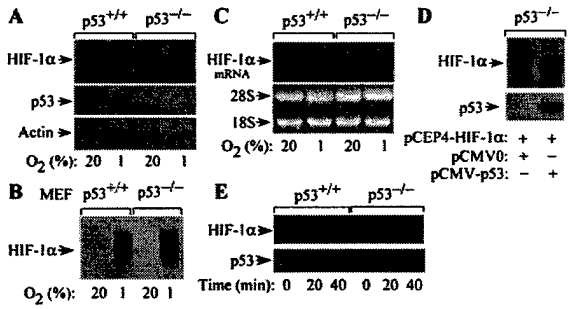

Figure 3. Effect of p53 on oxygen-regulated expression and stability of HIF-1α. (*A*) Immunoblot analysis of HIF-1α expression in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells cultured for 8 hr in 20%or 1%O_2. The blot was analyzed sequentially with monoclonal antibodies against HIF-1α (H1α67), p53 (DO-1), and β-actin. (*B*) Immunoblot analysis of HIF-1α expression in $p53^{+/+}$ and $p53^{-/-}$ MEFs cultured for 8 hr in 20% or 1% O_2. (*C*) Northern blot analysis of HIF-1α mRNA expression in $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells cultured as in *A*. (*D*). Immunoblot analysis of HIF-1α protein in $p53^{-/-}$ HCT116 cells cultured in 1% O_2 for 8 hr following cotransfection with pCEP4–HIF-1α and either pCMV–p53 or empty vector. The blot was analyzed sequentially with anti-HIF-1α and anti-p53 monoclonal antibodies. (*E*) Half-life of HIF-1α protein in $p53^{+/+}$ and $p53^{-/-}$ cells exposed to 100 μM cobalt chloride following addition of 100 μM cycloheximide. Lysates of cells harvested at the indicated time intervals were subject to immunoblot analysis of HIF-1α and p53 expression.

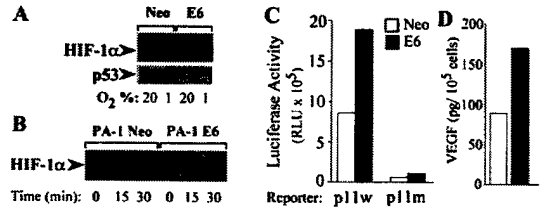

Figure 4. HPV E6 increases expression of HIF-1α and VEGF in response to hypoxia. (*A*) Immunoblot analysis of HIF-1α expression in PA-1 Neo or PA-1 E6 cells cultured for 8 hr in 20% or 1% O_2. (*B*) Half-life of HIF-1α protein in PA-1 Neo or PA-1 E6 cells exposed to 100 μM cobalt chloride following addition of 100 μM cycloheximide. Lysates of cells harvested at the indicated time intervals were subject to immunoblot analysis of HIF-1α expression. (*C*) Hypoxia-induced and HIF-1-dependent activation of VEGF-reporter activity in PA-1 Neo (open bars) and PA-1 E6 (solid bars) cells. Cells were cotransfected with either VEGF–p11w or VEGF–p11m and CMVβgal, exposed to 1% O_2 for 20 hr, and harvested for luciferase assays. The data represent the mean luciferase activity (normalized for β-gal activity) from three independent experiments. (*D*) ELISA of VEGF protein concentration in supernatant medium of PA-1 Neo (open bar) or PA-1 E6 (solid bar) cells incubated for 16 hr in 1% O_2.

with PA-1 Neo cells (Fig. 4C). Neither cell line exhibited significant transcription of the VEGF-p11m reporter. Consistent with the promotion of HIF-1-dependent VEGF transcription by E6 expression, exposure to 1% O_2 resulted in greater induction of VEGF protein expression in PA-1 E6 cells compared with PA-1 Neo cells (Fig. 4D).

p53 promotes ubiquitin-dependent of HIF-1α

To determine whether p53 interacts with HIF-1α in HCT116 cells, as previously demonstrated in MCF-7 cells (An et al. 1998), protein lysates from hypoxic $p53^{+/+}$ and $p53^{-/-}$ cells were immunoprecipitated with an anti-p53 or isotype control antibody, and the resulting immune complexes were subjected to immunoblot assays using an antibody against HIF-1α. HIF-1α was detected in immunoprecipitates derived from $p53^{+/+}$ cells but not $p53^{-/-}$ cells or immune complexes precipitated with the control antibody (Fig. 5A).

To determine whether p53 promotes ubiquitination of HIF-1α, $p53^{+/+}$ and $p53^{-/-}$ cells were cotransfected with an HIF-1α expression vector (pCEP4/HIF-1α) and a vector encoding hexahistidine-tagged ubiquitin (His_6-Ub) or the empty control vectors. Transfected cells were exposed to 1% O_2 for 4 hr in the presence of MG132, a peptide aldehyde inhibitor of the 20S proteasome. Aliquots of whole-cell extracts or His-tagged proteins isolated by affinity purification from cell lysates were subjected to immunoblot assays using an anti-HIF-1α monoclonal antibody (Fig. 5B). Immunoblot analysis of whole cell extracts of $p53^{+/+}$ cells detected a 120-kD protein corresponding to the apparent molecular mass of HIF-1α (Wang et al. 1995), as well as an additional series of slower migrating complexes. The higher molecular weight complexes represented polyubiquitinated forms of HIF-1α as they were also detected by immunoblot analysis of His-tagged proteins with an anti-HIF-1α monoclonal antibody. Compared with $p53^{+/+}$ cells, $p53^{-/-}$ cells transfected with vectors encoding HIF-1α and His_6-Ub demonstrated a higher level of unconjugated HIF-1α and a reciprocal reduction in polyubiquitinated HIF-1α (Fig. 5B). Introduction of a p53 expression vector (pCMV–p53) into $p53^{-/-}$ cells increased the proportion of HIF-1α that was ubiquitinated under hypoxic conditions (Fig. 5B).

Conjugation of Ub to proteins destined for degradation involves conversion of Ub to a high-energy thiol ester by the E1 Ub-activating enzyme followed by the transfer of activated Ub to the substrate via the activity of an E2 Ub-conjugating enzyme and an E3 Ub–protein ligase (for review, see Hershko and Ciechanover 1998). To confirm the requirement of the Ub-proteasome system for p53-mediated degradation of HIF-1α, we examined the effect of p53 on hypoxia-induced HIF-1α expression in the BALB/c 3T3-derived $ts20TG^R$ cell line, which harbors a thermolabile E1, or a derivative cell line (H38-5), in which the temperature-sensitive defect was corrected by introduction of the human E1 cDNA (Chowdary et al. 1994). $ts20TG^R$ and H38-5 cells were transfected with either an expression vector encoding human p53 or a control vector and transferred to hypoxic chambers (1% O_2) at either the permissive temperature (35°C) or the restrictive temperature (39°C). Transfection of p53 into $ts20TG^R$ cells resulted in reduced HIF-1α levels at 35°C but not at 39°C (Fig. 5C). However, E1-expressing H38-5 cells exhibited p53-mediated reduction of HIF-1α levels at both temperatures. Taken together, the data indicate that p53 limits hypoxia-induced expression of HIF-1α by promoting its ubiquitination and proteasomal degradation.

Whereas a single E1 is responsible for activation of ubiquitin, multiple E3 enzymes are responsible for specific selection of proteins destined for degradation. Because p53 induces the Mdm2 E3 Ub–protein ligase and is itself a target for Ub-mediated degradation via its interaction with Mdm2 (Momand et al. 1992; Barak et al. 1993; Wu et al. 1993; Haupt et al. 1997; Honda et al. 1997; Kubbutat et al. 1997), this raised the possibility that HIF-1α is recruited to Mdm2 via its interaction with p53. To test this hypothesis, protein lysates of $p53^{-/-}$ HCT116 cells that were transfected with either pCMV–p53 or empty vector and transferred to 1%O_2 for 6 hr were immunoprecipitated with anti-Mdm2 or isotype control antibody, and the resulting immune complexes were subjected to immunoblot assays using an antibody against HIF-1α. Anti-Mdm2 immunoprecipitates derived from cells transfected with p53 displayed significantly higher levels of coprecipitated HIF-1α protein compared to immune complexes derived from $p53^{-/-}$ HCT116 cells with the empty vector (Fig. 5D).

Amino acid residues Phe-19, Leu-22, and Trp-23 in the amino-terminal transactivation domain of p53 are critical for its interaction with Mdm2 (Lin et al. 1994). A p53 double mutant at residues 22 and 23 (p53 Gln22, Ser23) fails to interact with Mdm2 and is also transactivation

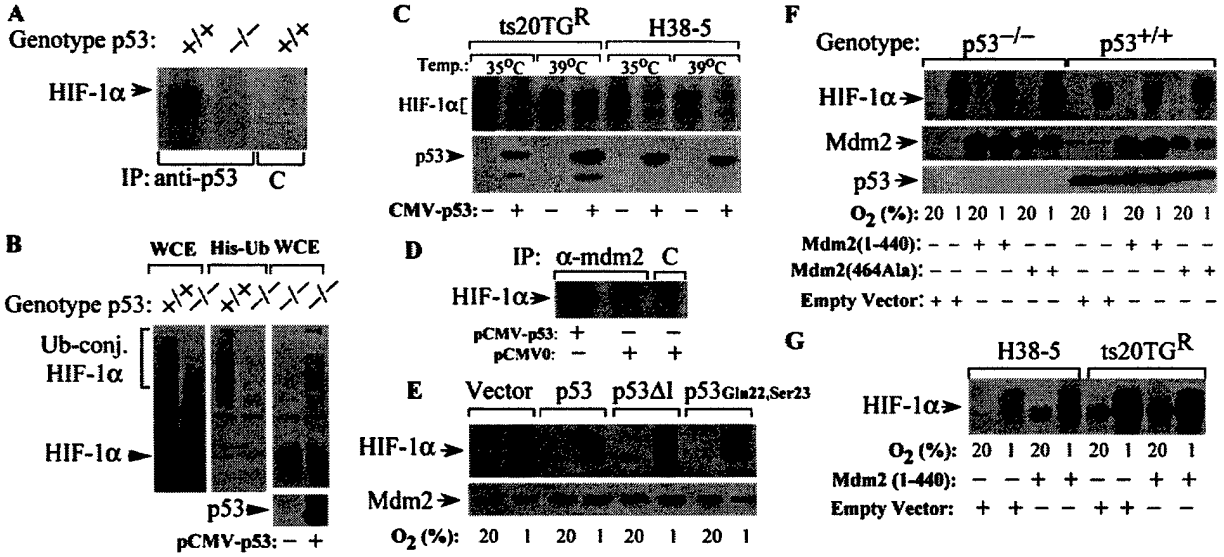

Figure 5. Effect of p53 expression on ubiquitin-mediated degradation of HIF-1α. (*A*) Interaction of p53 with HIF-1α. Lysates of $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells exposed to 1%O_2 for 8 hr were immunoprecipitated with either anti-p53 antibody or isotype control antibody (*C*) and the resultant immune complexes were subjected to immunoblot analysis with anti-HIF-1α monoclonal antibody. (*B*) Differential ubiquitination of HIF-1α in hypoxic $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells. Cells were cotransfected with pCMVβgal and pCEP4/HIF-1α with either MT107/His_6-Ub or empty vector (MT107), and cultured in 1%O_2 for 4 hr in the presence of 50 μM MG132. Aliquots of whole-cell extract (WCE) or His-tagged proteins purified from whole-cell lysates (His-Ub) were subjected to immunoblot analysis with anti-HIF-1β antibody. (*C*) Effect of p53 expression on HIF-1α protein levels in hypoxic $ts20TG_R$ and H38-5 cells. Cells transfected with pCMV–p53 or pCMVβgal were maintained at either 35°C or 39°C for 8 hr and exposed to 1%O_2 for an additional 8 hr at their respective temperatures. Whole-cell lysates were subjected to immunoblot analysis with anti-HIF-1α or anti-p53 antibodies. (*D*) Effect of p53 on complex formation between HIF-1α and Mdm2. Lysates of $p53^{-/-}$ HCT116 cells transfected with either pCMV–p53 or empty vector and transferred to 1%O_2 for 6 hr were immunoprecipitated with anti-Mdm2 or isotype control antibody, and the resulting immune complexes were subjected to immunoblot assays using an antibody against HIF-1α. (*E*) Effect of wild-type p53, p53ΔI, or p53Gln22,Ser23 on expression of HIF-1α in response to hypoxia. $p53^{-/-}$ HCT116 cells transfected with pCMVβgal and either pCMV–p53, pCB6 + p53ΔI, pCMV–p53Gln22,Ser23, or empty vector were exposed to 1%O_2 for 8 hr. Whole-cell lysates were subjected to immunoblot analysis with anti-HIF-1α or anti-Mdm2 antibodies. (*F*) Effect of dominant-negative (RING finger) mutants of Mdm2 on hypoxia-induced expression of HIF-1α. $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells transfected with vectors encoding human Mdm2 (1–440) (pCHDM1–440), Mdm2 (464Ala) (pCHDM464Ala), or pCMVβgal were exposed to 1%O_2 for 8 hr. Whole-cell lysates were subjected to immunoblot analysis with anti-HIF-1α, anti-p53, or anti-Mdm2 antibodies. (*G*) Effect of dominant-negative (RING finger deletion mutant) Mdm2 on p53-mediated inhibition of HIF-1α expression in $ts20TG^R$ and H38-5 cells. Cells cotransfected with pCMV–p53 and either pCHDM1–440 or empty vector were maintained at 39°C for 12 hr and then exposed to 20%or 1%O_2 for an additional 8 hr at 39°C. Whole-cell lysates were subjected to anti-HIF-1α immunoblot analysis.

deficient (Lin et al. 1994). A p53 mutant carrying a deletion of residues 13–19 (p53ΔI) is also unable to bind to Mdm2 but retains its transactivation function (Marston et al. 1995). To investigate whether p53 requires interaction with Mdm2 to mediate degradation of HIF-1α, $p53^{-/-}$ HCT116 cells were transfected with encoding either wt p53, p53 22-23, p53ΔI, or control vector and analyzed for HIF-1α expression under hypoxic conditions. In contrast to wild-type p53, the p53 mutants (p53ΔI or p53 Gln22, Ser23) or the control vector were unable to reduce the levels of HIF-1α (Fig. 5E).

The Ub–protein ligase function of Mdm2 is dependent on a RING finger domain (residues 434–490) at the carboxyl terminus (Honda et al. 1997). Mdm2 mutants with a deletion of the RING finger domain [Mdm2 (1–440)] or a substitution of a cysteine residue at position 464 to alanine [Mdm2 (464Ala)] are deficient in Ub–protein ligase function but retain the ability to bind p53, thereby behaving in a dominant negative manner (Kubbutat et al. 1999). Introduction of Mdm2 (1–440) or Mdm2 (464Ala) augmented hypoxia-induced HIF-1α levels in $p53^{+/+}$ HCT116 cells but did not significantly influence HIF-1α expression in hypoxic $p53^{-/-}$ HCT116 cells (Fig. 5F). To determine whether Mdm2 functions as an E3-ligase that mediates p53-induced degradation of HIF-1α, $ts20TG^R$ and H38-5 cells were cotransfected with expression vectors encoding wild-type p53 and either Mdm2 (1–440) or empty control vector and transferred to hypoxic chambers (1%O_2) at 39°C. Cotransfection of Mdm2 (1–440) increased hypoxia-induced HIF-1α expression in E1-proficient H38-5 cells coexpressing p53 to levels observed in E1-deficient $ts20TG^R$ cells (Fig. 5G). Together, the data in Figure 5 are consistent with a model in which p53 acts as a molecular chaperone that facilitates recognition and recruitment of HIF-1α for ubiquitination by Mdm2.

Enhancement of tumor angiogenesis in $p53^{+/+}$ cells by forced expression of HIF-1α

To determine whether p53-mediated degradation of HIF-1α contributes to the suppression of tumor angiogenesis and growth, $p53^{+/+}$ HCT116 cells were stably transfected with pCEP4/HIF-1α (HCT116–HIF-1α) (Fig. 6A). Under hypoxic conditions, stable transfectants overexpressing HIF-1α demonstrated significantly increased VEGF mRNA levels compared with the parental $p53^{+/+}$ cells (Fig. 6B). When inoculated into athymic nude mice, HCT116–HIF-1α cells established tumors with a shorter latency and exhibited a significant increase in tumor growth kinetics compared with the parental cells (Fig. 6C). Histologic evaluation and analyses of NMR maps, as described earlier, revealed a significant increase in blood vessel density, vascular volume (17.4 μl/g) and , (0.8 μl/g/min) in xenografts established from HCT116–HIF-1α cells compared with those derived from the parental $p53^{+/+}$ HCT116 cells (Fig. 6D).

Discussion

Recognition of the importance of angiogenesis for the growth and metastasis of cancers has raised fundamental questions regarding the molecular mechanisms of the angiogenic switch during tumor progression. The genetic alterations involved in tumorigenesis are also responsible for the phenotypic characteristics of cancer cells. The *p53* tumor suppressor gene is one of the most frequently mutated genes in human cancers (for review, see Levine 1997). In addition to p53 mutations, which occur in ~50% of all cancers (involving >50 tissue types), p53 is also inactivated by viral oncoproteins such the E6 protein of cervical cancer-associated HPV 16 and 18, adenovirus E1A, and SV40 large T antigen (for review, see Levine 1997). Our observations indicate that loss of p53 function, via somatic mutations or expression of viral oncoproteins, contributes to activation of the angiogenic switch during tumorigenesis.

In addition to identifying the loss of p53 as a discrete and potentially rate-limiting event in tumor angiogenesis, we define a novel mechanism by which p53 regulates the angiogenic switch. Our observations indicate that p53 inhibits hypoxia-induced expression of HIF-1α by facilitating its ubiquitination and subsequent degradation. This mechanism is distinct from the proposal that p53 inhibits HIF-1-mediated transactivation by competing for the p300 coactivator (Blagosklonny et al. 1998) and is analogous to the proposed role of the von Hippel-Lindau (VHL) tumor suppressor (Maxwell et al. 1999). As in the case of VHL (Maxwell et al. 1999), we demonstrate that p53 interacts with HIF-1α in vivo, as reported previously (An et al. 1998). In addition, we demonstrate for the first time that a tumor suppressor (p53) promotes the ubiquitin-mediated degradation of HIF-1α via recruitment of an E3 ubiquitin–protein ligase (Mdm2). Although ubiquitination is assumed to be the mechanism by which VHL affects HIF-1α degradation, our data provide the first direct evidence for this mechanism of tumor suppressor action. The constitutive stabilization of HIF-1α and the related HIF-2α protein) resulting from VHL loss of function may underlie the predisposition to highly angiogenic tumors in VHL disease, a rare hereditary cancer syndrome. Our findings indicate that deregulation of HIF-1α expression, leading to overexpression of VEGF, may contribute to the angiogenic switch conferred by inactivation of p53 in a broad array of human cancers. In accordance with this hypothesis, HIF-1α is frequently overexpressed in common human cancers and there is a statistically significant correlation between the presence of mutant p53 and HIF-1α overexpression (Zhong et al. 1999). Our findings suggest that increased HIF-1 activity resulting from loss of p53 function may contribute to the overexpression of VEGF that is observed in a wide variety of human cancers (for review, see Brown et al. 1996; Folkman 1997).

The angiogenic switch is regulated by changes in the relative balance between inducers and inhibitors of en-

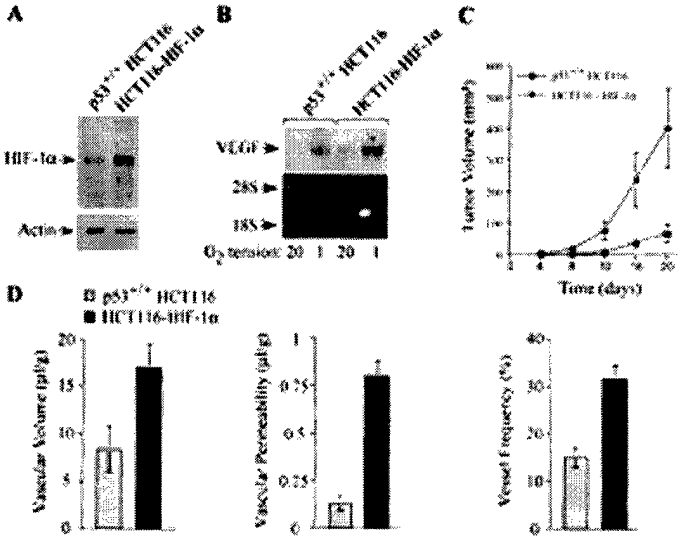

Figure 6. Increased tumor angiogenesis and growth in $p53^{+/+}$ cells with forced overexpression of HIF-1α. (*A*) Immunoblot analyses of HIF-1α protein levels in $p53^{+/+}$ HCT116 cells and $p53^{+/+}$ HCT116 cells stably transfected with a HIF-1α expression vector (HCT116–HIF-1α following exposure to 1% O_2 for 8 hr. (*B*) Northern blot analysis of VEGF mRNA levels in $p53^{+/+}$ HCT116 and HCT116–HIF-1α cells cultured for 16 hr in 20% or 1% O_2. (*C*) Growth of $p53^{+/+}$ HCT116 (blue ■) and HCT116–HIF-1α (red ▲) cells (2.5×10^6) injected subcutaneously into the flanks of athymic BALB/c nude mice. Values expressed represent mean ± S.E. of 12 xenografts of each cell type. (*D*) Quantification of vascular volume, permeability, and blood vessel density in $p53^{+/+}$ HCT116 (shaded bars) and HCT116–HIF-1α (solid bars) xenograft tumors. In vivo vascular volume and permeability of the tumors were determined by NMR analyses, and blood vessel frequency in stained sections of excised tumors was analyzed as described in Fig. 1.

dothelial cell proliferation and migration (for review, see Hanahan and Folkman 1996). The switch can be activated by increasing the levels of inducers, such as VEGF, and/or by reducing the concentration of inhibitors, such as thrombospondin-1 (TSP-1). The p53-mediated inhibition of VEGF expression demonstrated in this study, together with the previously reported ability of p53 to upregulate TSP-1 (Dameron et al. 1994), indicates that p53 provides dual functions that regulate angiogenesis. Thus, the loss of p53 function during tumorigenesis deregulates both arms of the balance, providing a potent stimulus for neovascularization and tumor progression.

In addition to loss-of-function mutations in tumor suppressor genes such as *p53* or *VHL*, oncogene activation is also capable of stimulating HIF-1 activity. Expression of the *v-Src* oncogene induces expression of HIF-1α protein, HIF-1 DNA-binding activity, and transcriptional activation of *VEGF* and enolase 1 (Jiang et al. 1997). A phosphatidylinositol 3-kinase/Akt pathway of HIF-1 activation may induce *VEGF* expression in *Ha-ras*-transformed cells (Mazure et al. 1997). Therefore, increased HIF-1 expression is associated with multiple genetic alterations that promote tumor angiogenesis. Because HIF-1 is also a key transcriptional activator of genes encoding glucose transporters and glycolytic enzymes (Iyer et al. 1998), these genetic alterations also contribute to the metabolic adaptation and enhanced survival of tumor cells in hypoxic microenvironments.

As p53 is an important mediator of DNA damage-induced apoptosis, the angiogenic phenotype conferred by inactivation of p53 in human cancers is frequently associated with resistance to conventional genotoxic anticancer agents (for review, see Lowe 1995). Because p53-deficient tumors remain dependent on angiogenesis for growth and metastasis, inhibition of angiogenesis may represent an effective therapeutic intervention (Boehm et al. 1997; Bergers et al. 1999). Recent studies indicate that inhibition of tumor-derived VEGF expression restricts angiogenesis and promotes vascular regression in experimental tumor models (Kim et al. 1993; Millauer et al. 1994, 1996; Warren et al. 1995; Goldman et al. 1998). Loss of HIF-1 activity is also associated with decreased angiogenesis and growth of tumor xenografts in nude mice (Jiang et al. 1997; Maxwell et al. 1997; Carmeliet et al. 1998; Ryan et al. 1998). By demonstrating that deregulation of HIF-1 underlies the increased expression of VEGF in p53-deficient cancers, our data provide further support for the hypothesis that inhibition of HIF-1 may abrogate the ability of such tumors to establish an adequate vascular supply and adapt their cellular metabolism to hypoxia, thereby curtailing their growth and metastasis.

Materials and methods

Cell lines and culture

The parental HCT116 human colon adenocarcinoma cell line, containing wild-type p53 ($p53^{+/+}$), and a p53-deficient derivative ($p53^{-/-}$) created by homozygous deletion via homologous recombination (Bunz et al. 1998), were a gift from Bert Vogelstein. $p53^{+/+}$ HCT116 cells were transfected with pCEP4/HIF-1α and a pool of stable transformants overexpressing HIF-1α (HCT116–HIF-1α) was selected in the presence of hygromycin (200 μg/ml). $p53^{+/+}$ or $p53^{-/-}$ HCT116 and HCT116–HIF-1α cells were maintained in McCoy's modified medium (Life Technologies, Inc.) supplemented with 10%fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5%CO_2. PA-1 ovarian teratocarcinoma cells stably transfected with pCMV–HPV16 E6 or pCMV–Neo, generated as described (Ravi et al. 1998), were maintained in Basal Eagle medium supplemented with 0.5 mg/ml G418, 10%FCS, and antibiotics (as described above) at 37°C and 5%CO_2. $p53^{+/+}$ and $p53^{-/-}$ MEFs (gift from Tyler Jacks, Massachusetts Institute of Technology, Cambridge, MA), were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10%FCS and antibiotics. The BALB/c 3T3-derived ts20TG^R or H38-5 cell lines (Chowdary et al. 1994) (gift from Harvey L. Ozer) were maintained at 35°C in DMEM supplemented with 10%fetal bovine serum and antibiotics. The permissive and nonpermissive temperatures for the ts20TG^R E1-mutant cell line are 35°C and 39°C, respectively. Cells were plated on 100-mm petri dishes and allowed to approach confluence. For hypoxic conditions, cells were placed in a modular incubator chamber and flushed with a gas mixture containing 1%O_2, 5%CO_2, and balance N_2 (Semenza and Wang 1992).

Growth of tumor xenografts in nude mice

HCT116 cells (2.5×10^4, 2.5×10^5, or 2.5×10^6) suspended in 0.1 ml of PBS were injected subcutaneously into the right ($p53^{+/+}$) or left ($p53^{-/-}$) hind legs or flanks of athymic BALB/c (nu/nu) mice. Tumor volumes were determined by external measurement in three dimensions using the equation $V = [L \times W \times H] \times /6$, where V = volume, L = length, W = width, and H = height. Care of experimental animals was in accordance with institutional animal care and use committee guidelines.

NMR analyses of in vivo vascular volume and permeability of tumor xenografts

Multislice maps of relaxation rates (T_1^{-1}) were obtained by a saturation recovery T_1 SNAPSHOT-FLASH imaging method (flip angle of 5°, echo time of 2 msec). Images of four slices (slice thickness of 1 mm) acquired with an in-plane spatial resolution of 125 μm (128 × 128 matrix, 16-mm field of view, NS = 8) were obtained for three relaxation delays (100 msec, 500 msec, and 1 sec) for each of the slices; 128 × 128 × 4 T_1 maps were acquired within 7 min. An M_o map with a recovery delay of 7 sec was acquired once at the beginning of the experiment. Images were obtained before intravenous administration of 0.2 ml of 60 mg/ml albumin–GdDTPA in saline (dose of 500 mg/kg) and repeated starting after the injection up to 32 min. Relaxation maps were reconstructed from data sets for three different relaxation times and the M_o data set on a pixel by pixel basis. At the end of the imaging studies, the animal was sacrificed, and 0.5 ml of blood was withdrawn from the inferior vena cava. Vascular volume and permeability product surface area (PS) maps were generated from the ratio of $\Delta(1/T_1)$ values in the images to that of blood. The slope of $\Delta(1/T_1)$ ratios versus time in each pixel was used to compute PS, whereas the intercept of the line at zero time was used to compute vascular volume. Thus, vascular volumes were corrected for permeability of the vessels. Volume and permeability values (mean ± S.E.) were computed for tumor xenografts established with HCT116

$p53^{+/+}$ (n = 4), HCT116 $p53^{-/-}$ (n = 5), and HCT116–HIF-1α (n = 2) cells.

Histologic analyses of blood vessel density in tumor xenografts

Five-micrometer sections prepared from paraffin-embedded tissue were stained with hemotoxin & eosin (H & E) and subjected to immunoperoxidase detection of endothelial cells using an anti-vWF antibody. A circular matrix of 25 random sampling points (per unit area) was superimposed on defined fields, and the points overlying a vessel were scored as a percentage of the total points.

Plasmids

Plasmids encoding human wild-type full-length p53 (pC53-SN; gift from Bert Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD), mutant p53 (pCB6+ p53ΔI) (Marston et al. 1994), p53 double mutants (pCMV–p53Gln22, Ser23) (Lin et al. 1994), human mutant Mdm2 (pCHDM1-440 and pCHD464Ala; provided by Karen Vousden) (Kubbutat et al. 1999), His_6–Ubiquitin (MT107–His_6-Ub; provided by Dirk Bohmann) (Musti et al. 1997), pCMV–HPV16 E6 (provided by Kathy Cho, Johns Hopkins University School of Medicine, Baltimore, MD), and HIF-1α (pCEP4–HIF-1α) (Forsythe et al. 1996; Jiang et al. 1996) have been described previously.

Analysis of VEGF reporter activity

Wild-type (p11w) and mutant (p11m) copies of the hypoxia response element from the *VEGF* gene cloned 5′ to a SV40-promoter–luciferase transcription unit were described previously (Forsythe et al. 1996). $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells and PA-1 Neo or PA-1 E6 cells were cotransfected (using Lipofectin) with either VEGF–p11w or VEGF–p11m and CMVβgal, with or without pCEP4/HIF-1α. Transfected cells were exposed to hypoxia (1%O_2) for 20 hr and harvested for β-gal and luciferase assays (Promega) in fixed protein aliquots. Luciferase activity was normalized for β-gal activity. The data represent the mean ± S.E. from three independent experiments.

Electrophoretic mobility shift assays of HIF-1 DNA-binding activity

Nuclear extracts (5 μg) prepared from $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells exposed to either 20%or 1%O_2 were incubated with ^{32}P-labeled double-stranded oligonucleotide probe containing a wild-type HIF-1 binding site and DNA/protein complexes were analyzed by polyacrylamide gel electrophoresis as described previously (Semenza and Wang 1992; Jiang et al. 1996). HIF-1 binding to the probe was confirmed by competition assays using 50 ng of either unlabeled wild-type oligonucleotide or a mutant oligonucleotide containing the same 3-bp substitution as in p11m (Semenza and Wang 1992; Forsythe et al. 1996).

Northern blot

VEGF and HIF-1α mRNA was assessed by Northern blot analyses of total RNA prepared from $p53^{+/+}$ or $p53^{-/-}$ HCT116 and HCT116–HIF-1α cells cultured for 16 hr in either 20%or 1%O_2. Total RNA (20-μg aliquots) was fractionated by 1.2%agarose–formaldehyde gel electrophoresis and transferred to nylon membranes. The blots were hybridized to probes for VEGF, HIF-1α, and β-actin mRNA using random primer labeling (Boehringer Mannheim) (Jiang et al. 1997).

ELISA

Quantikine (R & D Systems) was used to measure VEGF protein in supernatant medium of $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells and PA-1 Neo or PA-1 E6 cells cultured as described above for 16–32 hr.

Immunoblot analyses and immunoprecipation

Nuclear extracts or whole-cell lysates were prepared, fractionated by SDS-PAGE, transferred to PVDF membranes (Millipore, Bedford, MA), and immunoblotted with monoclonal antibodies against HIF-1α (H1α67; Novus Biologicals, Inc.) (Zhong et al. 1999), p53 (DO-1, Ab-6; Oncogene Research Products), Mdm2 (Ab-1; Oncogene Research Products), or β-actin (Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham). For analysis of protein interactions, whole-cell lysates were immunoprecipitated with antibodies against either p53 or Mdm2 or isotype control antibody, and the resultant immune complexes were subjected to immunoblot analysis with anti-HIF-1α monoclonal antibody H1α67.

Analysis of HIF-1α protein half-life and ubiquitin-dependent degradation

Cells exposed to 100 μM cobalt chloride for 4 hr were treated with 100 μM cycloheximide. Whole-cell extracts were prepared at intervals of 15–40 min and subjected to immunoblot analyses with anti-HIF-1α antibody. To assess ubiquitination of HIF-1α in hypoxic conditions, $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells were cotransfected (Lipofectin, Life Technologies, Inc.; 100-mm dishes) with 2 μg of pCMVβgal and 6μg each of pCEP4/HIF-1α and either MT107/His_6-Ub or empty vector (MT107) (Musti et al. 1997), and cultured in 1%O_2 for 4 hr in the presence of 50 μM MG132 (Peptides International, Inc.). Cells were lysed in buffer supplemented with 5 mM N-ethylmaleimide and 50 mM imidazole, as described (Ravi et al. 1998). Whole-cell extracts or His-tagged proteins [purified from 500 μg of whole cell–protein lysates (normalized to β-gal activity) using Talon Metal Affinity Resin (Clontech)] were subjected to SDS-PAGE and immunoblot analysis with anti-HIF-1α antibody. To analyze whether the effect of p53 on HIF-1α protein levels was dependent on ubiquitination, ts20TG^R and H38-5 cells were transfected (using Lipofectin) with pCMV–p53 and pCMVβgal, maintained at either 35°C or 39°C for 8 hr, and then exposed to 1%O_2 for an additional 8 hr at their respective temperatures. Whole-cell lysates were subjected to immunoblot analysis with anti-HIF-1α or anti-p53 antibodies.

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Appendix 2: Reprint of publication: (Page Numbers 24-32)

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Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB

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TRAIL (tumour-necrosis factor-related apoptosis ligand or Apo2L) triggers apoptosis through engagement of the death receptors TRAIL-R1 (also known as DR4) and TRAIL-R2 (DR5). Here we show that the c-Rel subunit of the transcription factor NF-κB induces expression of TRAIL-R1 and TRAIL-R2; conversely, a transdominant mutant of the inhibitory protein IκBα or a transactivation-deficient mutant of c-Rel reduces expression of either death receptor. Whereas NF-κB promotes death receptor expression, cytokine-mediated activation of the RelA subunit of NF-κB also increases expression of the apoptosis inhibitor, Bcl-x_L, and protects cells from TRAIL. Inhibition of NF-κB by blocking activation of the IκB kinase complex reduces Bcl-x_L expression and sensitizes tumour cells to TRAIL-induced apoptosis. The ability to induce death receptors or Bcl-x_L may explain the dual roles of NF-κB as a mediator or inhibitor of cell death during immune and stress responses.

Apoptosis has an essential role in embryogenesis, adult tissue homeostasis and the cellular response to stressful stimuli, such as DNA damage, hypoxia or aberrations in cell-cycle progression[1]. Increased apoptosis is involved in the pathogenesis of diverse ischaemic, degenerative and immune disorders[2]. Conversely, genetic aberrations that render cells incapable of executing their suicide program promote tumorigenesis and underlie the observed resistance of human cancers to genotoxic anticancer agents[3]. Unravelling mechanisms to unleash the apoptotic program in tumour cells might aid the design of effective therapeutic interventions against resistant human cancers.

The molecular machinery of cell death comprises an evolutionarily conserved family of cysteine aspartate proteases (caspases)[4]. Caspases can be activated by the engagement of death receptors belonging to the tumour-necrosis factor (TNF) receptor gene superfamily[5], such as TNFR1, CD95 (Fas), TRAIL-R1 (DR4)[6] and TRAIL-R2 (DR5, TRICK2, KILLER)[7–13], by their respective cognate 'death ligands', TNF-α, CD95L (Apo1L) and TRAIL (also known as Apo2L)[14,15]. TRAIL induces apoptosis in several tumour cell lines, including those that resist chemotherapeutic agents or ionizing radiation because of inactivating mutations of the p53 tumour suppressor gene[16–20].

TRAIL-R1 and TRAIL-R2 are type I transmembrane proteins containing cytoplasmic sequences, termed 'death domains', that recruit adaptor proteins and activate caspases[16]. Two other TRAIL receptors, TRAIL-R3 (TRID/DcR1) and TRAIL-R4 (TRUNDD/DcR2), have extracellular domains similar to TRAIL-R1 and TRAIL-R2, but lack a functional cytoplasmic death domain[7,8,21–24]. TRAIL-R3 and TRAIL-R4 may serve as 'decoys' that compete with TRAIL-R1/TRAIL-R2 for binding to TRAIL, and overexpression of either protein confers protection against TRAIL-induced death[7,8].

The NF-κB family of dimeric transcription factors is important in modulating cell survival during stress and immune responses[25]. NF-κB protects cells from apoptosis[26–31] by promoting expression of survival factors, such as members of the inhibitor of apoptosis (IAP) family (c-IAP1, c-IAP2, XIAP)[32] and the Bcl-2 homologues, Bfl-1/A1 (refs 33, 34) and Bcl-x_L (ref. 35). In contrast, much evidence highlights an apparently paradoxical pro-apoptotic role for NF-κB[36–39]. These observations raise the possibility that κB sites in pro- or anti-apoptotic genes may exhibit different preferences for particular subunits comprising the NF-κB dimer, and that NF-κB may have signal-specific effects on cell survival.

Here we show that the RelA and c-Rel subunits of NF-κB are critical determinants of the expression of death receptors and survival genes that modulate TRAIL-induced apoptosis. The signal-specific activation of dimers that induce expression of either death receptors or survival genes might explain how NF-κB adopts either of its dual personalities as a mediator or inhibitor of cell death during immune and cellular stress responses. The identification of NF-κB as a key determinant of cellular susceptibility to TRAIL may have important implications for anticancer therapy.

Results

Subunit-specific effects of NF-κB on death receptor expression and on sensitivity to TRAIL. NF-κB exists in almost all cell types in an inactive cytoplasmic complex with an inhibitory protein, IκB. Signal-dependent phosphorylation and ubiquitin-mediated degradation of IκB by IκB kinases (IKKs) releases the active complex, which functions in transcriptional regulation of target genes after nuclear translocation[25]. Trimerization of TNFR1 by TNF-α leads to degradation of IκB and activation of NF-κB. Mouse embryonic fibroblasts (MEFs) stably transduced with a retrovirus carrying a combined amino- (residues 32 and 36) and carboxy-terminal PEST sequence phosphorylation mutant of IκBα (IκBαM)[28] show reduced basal and TNF-α-inducible κB DNA-binding activity and lower expression of TRAIL-R2 messenger RNA compared with wild-type MEFs carrying a control vector (Fig. 1a, b).

The subunits of NF-κB are known to exhibit different preferences for variations of the 10-base-pair (bp) consensus sequence

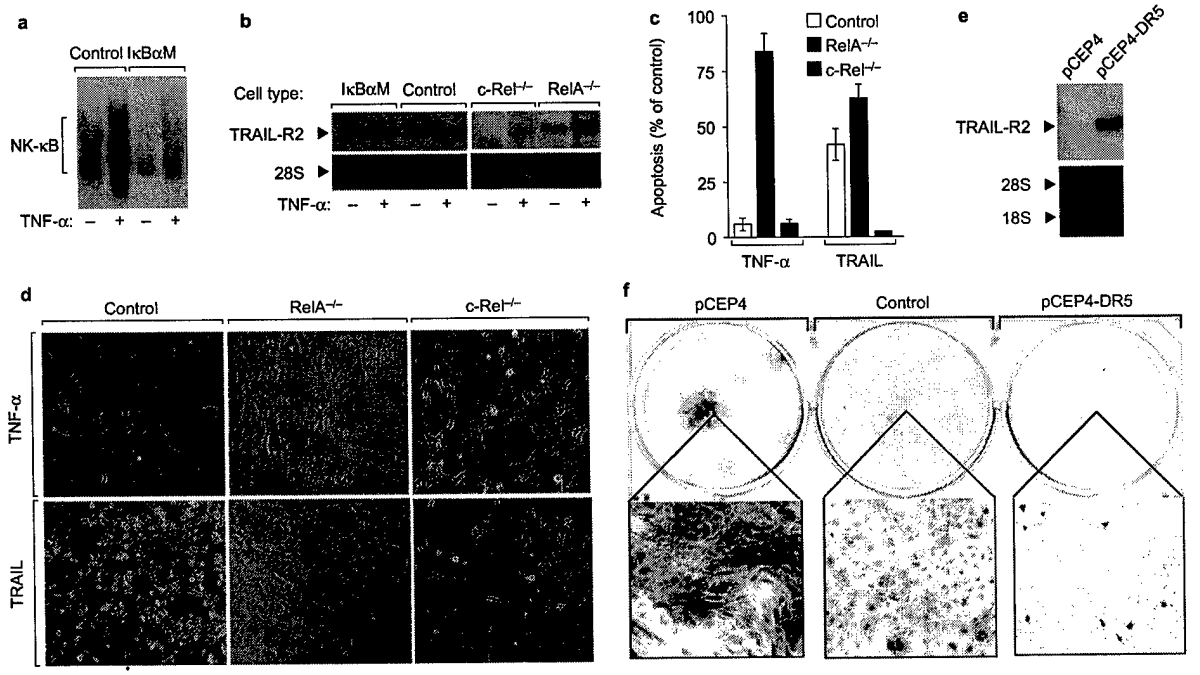

Figure 1 **Subunit-specific effects of NF-κB on death receptor expression and sensitivity to TRAIL. a**, Inhibition of NF-κB by a transdominant mutant IκBα (IκBαM). MEFs stably transduced with a plasmid encoding IκBαM and the empty vector pLXSN[28] (control) were incubated with TNF-α (100 ng ml^{-1}, 1 h) or left untreated. Nuclear extracts were analysed for NF-κB DNA-binding activity by EMSA. **b**, Basal and TNF-α-inducible expression of TRAIL-R2 mRNA in RelA$^{-/-}$, c-Rel$^{-/-}$, IκBαM-expressing, and wild-type mouse fibroblasts carrying an empty vector (control). **c**, **d**, Effect of deficiency of either RelA or c-Rel on TNF-α- or TRAIL-induced cell death. RelA$^{-/-}$, c-Rel$^{-/-}$ and wild-type mouse fibroblasts were exposed to either TNF-α (100 ng ml^{-1}) or recombinant human TRAIL (100 ng ml^{-1}; with enhancer antibody) for 24 h. Data (mean ± s.d.) shown in **c** are the percentage of apoptotic nuclei among total nuclei counted (n = 3). Representative photomicrographs illustrating the cytotoxicity of TRAIL are shown in **d**. **e**, Expression of TRAIL-R2 in c-Rel$^{-/-}$ mouse fibroblasts transfected with either pCEP4-DR5 or empty pCEP4 vector. **f**, Susceptibility of c-Rel-deficient cells to TRAIL-R2-induced death. Photomicrographs depict crystal-violet-stained colonies of c-Rel$^{-/-}$ mouse fibroblasts selected for growth in hygromycin B after transfection with either pCEP4-DR5 or empty pCEP4 vector. Cells from an untransfected control population were maintained in hygromycin-free media (control). Similar observations were made in RelA$^{-/-}$ and wild type mouse fibroblasts (data not shown).

(5′-GGGGYNNCCY-3′) in particular target genes[25]. We therefore analysed the role of specific subunit(s) of NF-κB on expression of TRAIL-R2 mRNA levels in RelA-deficient (RelA$^{-/-}$)[26], c-Rel-deficient (c-Rel$^{-/-}$)[40] and wild-type mouse fibroblasts. Whereas TNF-α-inducible expression of TRAIL-R2 mRNA was evident in RelA$^{-/-}$ fibroblasts, this induction was markedly diminished in c-Rel$^{-/-}$ fibroblasts (Fig. 1b).

We distinguished the effects of RelA and c-Rel on cell survival by examining the response of RelA$^{-/-}$, c-Rel$^{-/-}$ or wild-type mouse fibroblasts to either TNF-α or TRAIL. RelA$^{-/-}$ fibroblasts were highly sensitive to TNF-α-mediated cell death, but c-Rel$^{-/-}$ fibroblasts, akin to their wild-type counterparts, remained relatively resistant to such treatment (Fig. 1c, d). Whereas RelA$^{-/-}$ and wild-type fibroblasts were both susceptible to TRAIL-induced apoptosis, c-Rel$^{-/-}$ fibroblasts were almost completely resistant to TRAIL (Fig. 1c, d). c-Rel$^{-/-}$ cells were resistant to TRAIL, but they failed to yield any viable clones after transfection with an expression vector encoding TRAIL-R2 (pCEP4/DR5)[10] (Fig. 1e, f). The resistance of c-Rel$^{-/-}$ cells to TRAIL-induced death seems therefore to result from their deficiency in death receptor expression rather than inhibition of intracellular death signalling. These results suggest that, in contrast to the protection conferred by RelA against TNF-α-induced death, c-Rel mediates the inducible expression of death receptors for TRAIL.

NF-κB c-Rel contains an N-terminal 300-residue conserved region known as the Rel homology domain (RHD), which mediates dimerization and nuclear localization, and a variable C-terminal domain, which is responsible for transactivation. To examine directly the effect of c-Rel or RelA on death receptor expression and sensitivity to TRAIL, c-Rel (CCR), a c-Rel truncation mutant lacking the C-terminal transactivation domain (Δc-Rel; CCR-H) or RelA were conditionally expressed in HeLa cells using a tetracycline-regulated system[33,41] (Fig. 2a). The c-Rel, truncated c-Rel or RelA genes were expressed under control of the tTA fusion activator, comprising the *Escherichia coli* tetracycline repressor and the activation domain of the VP16 protein of herpes simplex virus. Stable cell clones carrying either c-Rel (CCR43) or Δc-Rel (CCR-H5) were subjected to immunoblot analysis using an antibody against the RHD of chicken c-Rel. Removing tetracycline from the culture medium for 48 h resulted in induction of either c-Rel in CCR43 cells or the faster migrating Δc-Rel mutant in CCR-H5 cells (Fig. 2b).

Electrophoretic mobility shift assays with double-stranded oligonucleotides containing a palindromic κB site were performed using nuclear protein derived from CCR43 or CCR-H5 cells maintained in the presence or absence of tetracycline for 48 h. CCR43 cells showed increased κB DNA-binding activity in response to withdrawal of tetracycline, and the DNA-bound complex was supershifted with an anti-c-Rel antibody but not with an antibody against RelA (Fig. 2c). Although the inducible c-Rel is active in binding c-Rel-responsive κB motifs, the transactivation-deficient mutant Δc-Rel competes with endogenous c-Rel for κB binding, thereby behaving in a dominant-negative manner (Fig. 2c)[33,41].

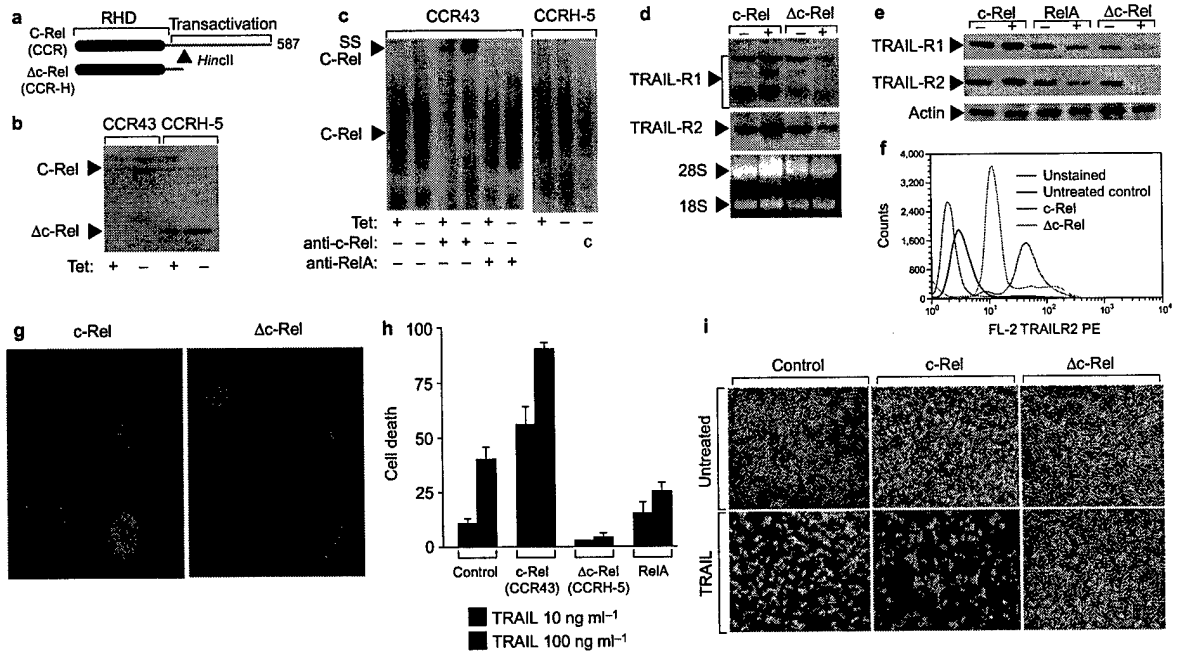

Figure 2 **Effect of inducible expression of c-Rel, ΔRel or RelA on death receptor expression and sensitivity to TRAIL.** **a**, Representation of full-length c-Rel (CCR) and Δc-Rel (CCR-H), a c-Rel mutant that contains a stop codon at the unique *Hin*cII site of c-Rel. **b**, Immunoblot analyses of expression of c-Rel and Δc-Rel in HeLa (HtTA-1) cell clones stably transfected with c-Rel (CCR43) or Δc-Rel (CCR-H5), respectively (in the presence or absence of tetracycline for 48 h). **c**, EMSA of c-Rel-specific DNA-binding activity in nuclear extracts of CCR43 and CCR-H5 cells maintained in the presence or absence of tetracycline (Tet) for 48 h. Supershift (SS) analysis of DNA–protein complexes was performed with anti-c-Rel and anti-RelA antibodies. Competition of the Δc-Rel-induced DNA–protein complex with unlabelled c-Rel-specific oligonucleotides is shown (lane 'c'). **d**, Northern blot analyses of TRAIL-R1 and TRAIL-R2 mRNA in cells maintained in the presence (uninduced, –) or absence (induced, +) of tetracycline for 48 h. **e**, Western blot analyses of the effect of induced expression of c-Rel or Δc-Rel on expression of TRAIL-R1 and TRAIL-R2 protein. **f**, Flow cytometric analysis of the effect of induced expression of c-Rel or Δc-Rel on IR-induced cell surface expression of TRAIL-R2 in HeLa cells. (Unstained controls received secondary antibody alone; untreated controls received no IR.) **g**, Confocal microscopic examination of TRAIL-R2 immunofluorescence in HeLa cells induced to express either c-Rel or Δc-Rel for 48 h. **h**, HeLa (HtTA-1) cell clones stably transfected with either c-Rel (CCR43), Δc-Rel (CCR-H5), or RelA were maintained in the absence of tetracycline for 48 h (to induce gene expression) and then exposed to TRAIL (10–100 ng ml^{-1}; enhancer antibody 2 μg ml^{-1}) or left untreated for another 24 h. Data represent the percentage survival (viable/[apoptotic + viable]) in each cell population (mean ± s.d.) from three independent experiments. **i**, Representative photomicrographs illustrating the cytotoxic effects of TRAIL (100 ng ml^{-1}) on c-Rel (CCR43) cells maintained in the presence of tetracycline (control), or in c-Rel (CCR43) and Δc-Rel (CCR-H5) cells induced to express c-Rel or Δc-Rel, respectively, by culture in tetracycline-free medium for 48 h.

Northern blot analysis showed that c-Rel promotes the expression of death receptors at a transcriptional level, but Δc-Rel interferes with this induction (Fig. 2d). Induction of c-Rel in CCR43 cells resulted in increased protein expression of both TRAIL-R1 (2.2-fold induction relative to an actin control) and TRAIL-R2 (2.6-fold induction) (Fig. 2e). In contrast, induction of the dominant-negative transactivation mutant Δc-Rel in CCR-H5 cells inhibited protein expression of either TRAIL-R1 (2.4-fold repression) or TRAIL-R2 (3.2-fold repression) (Fig. 2e). Flow cytometric analyses confirmed that inducible expression of cell-surface TRAIL-R2 was greater in cells expressing c-Rel compared with cells expressing Δc-Rel (Fig. 2f). Confocal microscopy showed relatively greater immunofluorescent labelling of TRAIL-R2 in the cytoplasm of cells induced to express c-Rel compared with cells forced to express Δc-Rel (Fig. 2g).

Induction of c-Rel by removing tetracycline resulted in a dose-dependent increase in the sensitivity of CCR43 cells to TRAIL-induced death (Fig. 2h, i). By contrast, expression of Δc-Rel by removing tetracycline in CCR-H5 cells rendered these cells relatively resistant to TRAIL (Fig. 2h, i). Consistent with its induction of survival factors, induced expression of RelA reduced sensitivity to TRAIL (Fig. 2h).

NF-kB-induces expression of TRAIL-R2 and TRAIL-mediated tumour cell radiosensitization independently of p53. The cellular response to DNA damage inflicted by genotoxic anticancer agents is modulated by the product of the p53 tumour suppressor gene—a transcription factor that promotes expression of TRAIL-R2/DR5 (ref. 10). As NF-κB has been implicated in p53-mediated cell death[38], we thought that p53 might be required for NF-κB-induced expression of TRAIL-R2. We therefore examined the effect of p53 genotype on the basal, TNF-α- and DNA-damage-induced activation of NF-κB and expression of death receptors in isogenic cell lines that differ only in p53 status.

The effect of TNF-α on expression of TRAIL-R2/DR5 was examined in MEFs of wild-type and p53$^{-/-}$ genotypes. Expression of TRAIL-R2/DR5 was impaired in c-Rel$^{-/-}$ cells (Fig. 1b), but *p53*$^{-/-}$ cells exhibited normal basal and TNF-α-inducible expression of TRAIL-R2 mRNA (Fig. 3a), indicating that NF-κB mediates TNF-α-induced expression of TRAIL-R2 in a p53-independent fashion. The parental HCT116 line, containing wild-type p53 (*p53*$^{+/+}$), and a p53-deficient derivative (*p53*$^{-/-}$), created by homozygous deletion of endogenous *p53* genes through homologous recombination[42], also showed equivalent basal levels of TRAIL-R2 mRNA (Fig. 3d). *p53*$^{+/+}$ or *p53*$^{-/-}$ HCT116 cells showed an equivalent reduction in

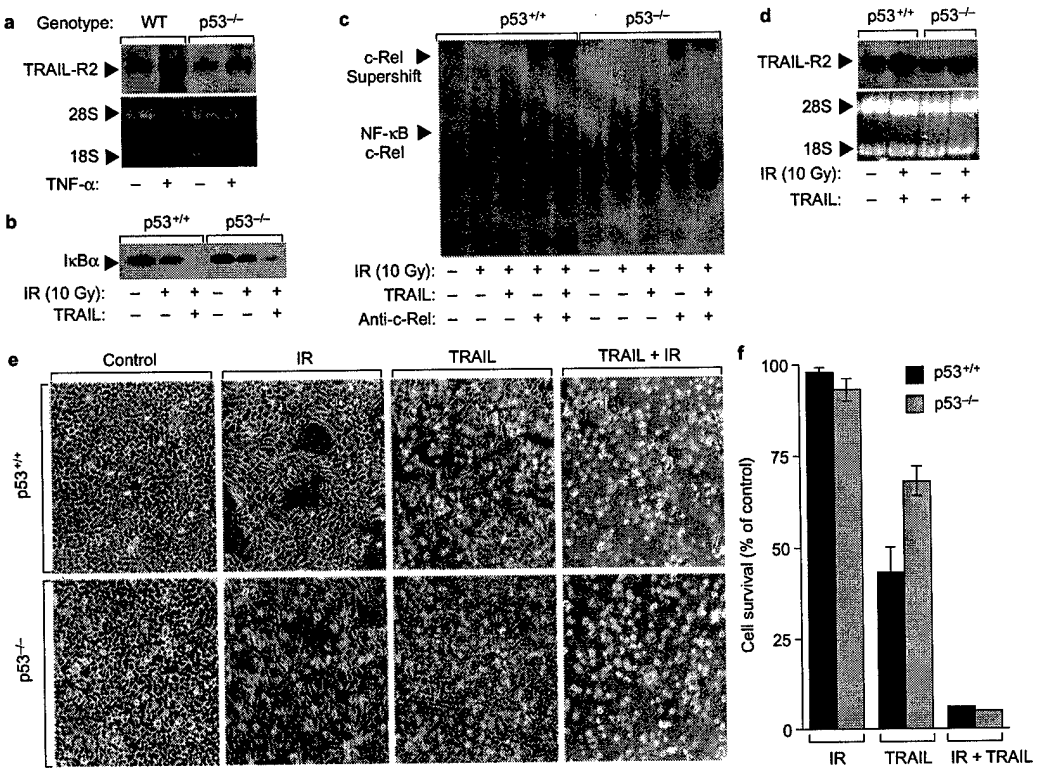

Figure 3 **NF-κB-induced expression of TRAIL-R2 and TRAIL-mediated radiosensitization independent of p53.** **a**, Northern blot analyses of the effect of TNF-α on TRAIL-R2 mRNA levels in wild-type (WT) and p53 $^{-/-}$ MEFs. **b**, Western blot analyses of IκBα expression in p53$^{+/+}$ or p53$^{-/-}$ HCT116 cells exposed to irradiation (10 Gy) in the presence or absence of TRAIL (100 ng ml^{-1} + enhancer antibody 2 μg ml^{-1}). **c**, EMSA of κB-specific DNA-binding activity in nuclear extracts of p53$^{+/+}$ or p53$^{-/-}$ HCT116 cells exposed to irradiation (10 Gy) in the presence or absence of TRAIL (100 ng ml^{-1} + enhancer antibody 2 μg ml^{-1}). Supershift (SS) analysis of DNA–protein complexes was performed an anti-c-Rel specific antibody. **d**, Western bot analyses of TRAIL-R2 expression in irradiated p53$^{+/+}$ or p53$^{-/-}$ HCT116 cells exposed to TRAIL (100 ng ml^{-1} + enhancer antibody 2 μg ml^{-1}). **e**, **f**, Representative photomicrographs illustrating the effects of either IR (10 Gy), TRAIL (100 ng ml^{-1} + enhancer antibody 2 μg ml^{-1}), and IR + TRAIL on survival of p53$^{+/+}$ or p53$^{-/-}$ HCT116 cells. Data in **f** represent the percentage survival (viable/[apoptotic + viable]) in each cell population (mean ± s.d.) from three independent experiments

IκBα levels and elevation in κB/c-Rel DNA-binding activity in response to irradiation, and irradiation-induced κB DNA-binding was augmented by exposure to TRAIL in both cell types (Fig. 3b, c). Exposure to ionizing radiation (IR) and TRAIL resulted in an equivalent elevation of TRAIL-R2 mRNA in both *p53*$^{+/+}$ and *p53*$^{-/-}$ HCT116 cells (Fig. 3d); therefore, IR-induced expression of TRAIL-R2 in cells exposed to TRAIL was analogous to its p53-independent expression after treatment with TNF-α.

To examine whether the combination of IR with TRAIL can override the radioresistance of HCT116 cells, we exposed *p53*$^{+/+}$ or *p53*$^{-/-}$ HCT116 cells to IR (10 Gy), TRAIL (100 ng ml^{-1}) or both. Although both *p53*$^{+/+}$ and *p53*$^{-/-}$ HCT116 cells were resistant to IR-induced apoptosis, exposure to IR resulted in augmentation of TRAIL sensitivity in both cell types, such that either cell population was eliminated within 48 h of treatment (Fig. 3e, f). Together, these data indicate that IR can induce NF-κB-mediated expression of death receptors and augment TRAIL-induced death of both *p53*$^{+/+}$ and *p53*$^{-/-}$ tumour cells. These data have potentially important implications for the treatment of p53-deficient human cancers by TRAIL-mediated radiosensitization.

The RelA subunit of NF-κB induces Bcl-x_L and protects cells from TRAIL/death-receptor-induced apoptosis. We investigated whether the differential activation of c-Rel- and/or RelA-containing dimers of NF-κB in response to physiological signals (immune activation by ligation of CD40) or stressful stimuli (DNA damage) influences the expression of death receptors and sensitivity to TRAIL.

Irradiation of B cells activated κB DNA-binding activity in electrophoretic mobility shift assays (EMSAs), using a c-Rel consensus binding site as an oligonucleotide probe, and the IR-induced DNA–protein complex was supershifted with an anti-c-Rel antibody (Fig. 4a). Although irradiation of wild-type B lymphocytes resulted in induction of TRAIL-R2 mRNA, IR-inducible levels of TRAIL-R2 were diminished in B cells from c-Rel$^{-/-}$ mice (Fig. 4b, c). Ligation of IR-induced TRAIL-R2 with TRAIL resulted in apoptosis of Bcl-2-overexpressing B lymphocytes (from TgN(Bcl-2) mice), which are otherwise relatively resistant to IR[43] (Fig. 4d).

Stimulation of resting mouse B lymphocytes with a monoclonal antibody against CD40 also resulted in activation of κB DNA-binding activity in EMSAs (Fig. 4a). The slower migrating DNA–protein complex was supershifted by an anti-c-Rel antibody that does not recognize RelA (Fig. 4a, lane 4), whereas a faster migrating complex was supershifted with an anti-RelA-specific antibody (Fig. 4, lane 3). CD40-mediated activation of c-Rel also induced TRAIL-R2 expression (Fig. 4b), but (unlike IR) it protected lymphocytes from TRAIL-induced death (Fig. 4d). Either CD40 ligation or IR activated c-Rel, but RelA-induced transcriptional activation of a HIV-CAT reporter (driven by two κB sites contained

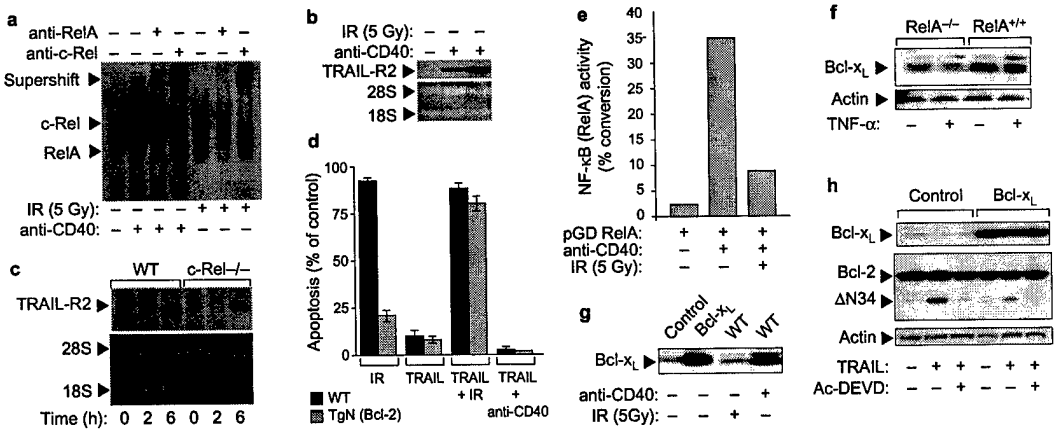

Figure 4 **The RelA subunit of NF-κB induces Bcl-x_L and protects cells from TRAIL/death receptor-induced apoptosis. a**, NF-κB DNA-binding activity in nuclear extracts of primary mouse B lymphocytes exposed to either anti-CD40 antibody (10 μg ml^{-1} for 16 h) or ionizing radiation (IR; 5 Gy). Supershift (SS) analysis of DNA–protein complexes was performed with anti-c-Rel- or anti-RelA-specific antibodies. **b**, Northern blot analysis of TRAIL-R2/DR5 expression in primary mouse B cells exposed to either anti-CD40 antibody or IR (5 Gy). **c**, Northern blot analysis of IR-induced expression of TRAIL-R2 in primary mouse B cells from wild-type (WT) or c-Rel$^{-/-}$ mice. **d**, Effect of IR (5 Gy), TRAIL (100 ng ml^{-1} + enhancer antibody 2 μg ml^{-1}), IR + TRAIL, or anti-CD40 antibody + TRAIL on survival of mouse B lymphocytes from WT or TgN(Bcl-2) mice. Data (mean ± s.d.) are the percentage apoptosis relative to untreated controls (n = 3). **e**, RelA-mediated HIV-CAT expression in activated B lymphocytes in response to CD40 or IR. **f**, Immunoblot analyses of basal or TNF-α-induced expression of Bcl-x_L in RelA$^{+/+}$ or RelA$^{-/-}$ fibroblasts. **g**, Expression of Bcl-x_L in mouse B cells in response to CD40 ligation or IR. HL-60-Neo (Control) or Bcl-x_L-overexpressing HL-60 (Bcl-x_L) cells were used as controls. **h**, Inhibition of caspase-3-mediated cleavage of Bcl-2 and TRAIL-induced death by expression of Bcl-x_L. HL-60-Neo (Control) or HL-60-Bcl-x_L (Bcl-x_L) cells were exposed to TRAIL (100 ng ml^{-1}) with or without pretreatment with Ac-DEVD-CHO (300 μM) and analysed for expression of Bcl-x_L and Bcl-2 12 h later. The full-length Bcl-2 (26K) and the Bcl-2 cleavage product (23K; ΔN34) are indicated. Percentage of each cell population that underwent apoptosis after 24 h: HL-60-Neo, 80 ± 5%; HL-60-Bcl-x_L cells, 27 ± 3%.

in the long-terminal repeat) was increased by anti-CD40 treatment but not by exposure to IR (Fig. 4e). This suggested that co-activation of RelA by CD40 ligation might inhibit TRAIL-induced apoptosis through RelA-induced expression of survival factor(s).

Compared with RelA$^{+/+}$ cells, RelA$^{-/-}$ cells exhibited reduced basal and TNF-α-inducible expression of the apoptosis inhibitor, Bcl-x_L (Fig. 4f). As Bcl-x_L expression in resting B cells was increased markedly in response to anti-CD40 (Fig. 4g), we investigated whether Bcl-x_L could inhibit TRAIL-induced death. Exposure of HL-60 cells (expressing wild-type Bcl-2; relative molecular mass (M_r) 26,000 (26K)) to TRAIL (100 ng ml^{-1}) resulted in the death of more than 80 ± 5% of the population within 24 h of treatment. This was associated with the appearance of a caspase-3-dependent 23K Bcl-2 cleavage product (Fig. 4h), previously identified as a C-terminal fragment (ΔN34; cleaved at Asp34) that lacks the loop domain and functions as a Bax-like death effector[44]. Stable transfection of a vector encoding Bcl-x_L into HL-60 cells inhibited caspase-3-dependent cleavage of Bcl-2 (Fig. 4h) and reduced TRAIL-induced apoptosis (27 ± 3% death of the total population at 24 h). Therefore, the reduction of TRAIL-induced apoptosis of B cells in the presence of anti-CD40 (despite c-Rel-mediated expression of TRAIL-R2) reflects the dominant protective effect of Bcl-x_L induced through the co-activation of RelA in activated B cells.

Together, these results illustrate the biological significance of NF-κB activity in regulating expression of both the death receptors and survival factors that determine cellular sensitivity to TRAIL. Our observations suggest that IR-induced NF-κB-mediated induction of death receptors can synergize with TRAIL to eliminate B cells overexpressing Bcl-2—a finding that may have implications for the treatment of resistant tumours, such as human follicular lymphomas. Our studies also indicate that RelA-mediated expression of Bcl-x_L may be responsible for the resistance of CD40-activated or transformed B cells to apoptotic signals transduced by death receptors.

Inhibition of NF-κB by blocking activation of the IKK complex sensitizes tumour cells to TRAIL. To determine the physiological significance of NF-κB in both the regulation of death receptor signalling and the sensitivity of tumour cells to TRAIL, we examined the effect of recombinant heregulin β1 (HRG β1), a ligand that induces HER-2/neu (c-erbB2)-mediated activation of NF-κB[45]. Exposure of MCF-7 human breast cancer cells to HRG β1 increased κB DNA-binding activity in EMSAs (Fig. 5a), and increased expression of TRAIL-R1 (4.2-fold induction relative to an actin control) and TRAIL-R2 (3.0-fold induction) (Fig. 5b). However, exposure of MCF-7 cells to HRG β1 also promoted the expression of Bcl-x_L (3.4-fold induction), and rendered them relatively resistant to TRAIL (Fig. 5d, e).

Activation of NF-κB requires the phosphorylation and ubiquitin-mediated degradation of IκBα by the IKK complex, which contains two kinases (IKK-α and IKK-β), and the regulatory protein NEMO (NF-κB essential modifier)[46]. A cell-permeable peptide (NEMO-binding domain (NBD) peptide) that blocks the interaction of NEMO with the IKK complex inhibits cytokine-induced NF-κB activation[46]. The anti-inflammatory agent, acetyl salicylic acid (aspirin; ASA), also specifically inhibits the activity of IKK-β[47].

Inhibiting activation of the IKK complex by either ASA or the wild-type NBD peptide prevented HRG β1-induced loss of IκBα or activation of NF-κB (Fig. 5a, b). Exposure of MCF-7 cells to either ASA or wild-type NBD (but not a mutant NBD peptide) prevented HRG β1 from either inducing expression of TRAIL-R1, TRAIL-R2 or Bcl-x_L (Fig. 5b, c). Exposure to either ASA or wild-type NBD (but not mutant NBD) inhibited HRG β1-mediated protection of MCF-7 cells from TRAIL-induced apoptosis (Fig. 5d, e). These data indicate that NF-κB promotes expression of both death receptors for TRAIL and Bcl-x_L, a protein that blocks death signals transduced by TRAIL. The dominant anti-apoptotic effect of Bcl-x_L allows NF-κB-activating cytokines, such as HRG β1, to confer protection against TRAIL. Conversely, inhibition of NF-κB after death receptor ligation can sensitize tumour cells to TRAIL.

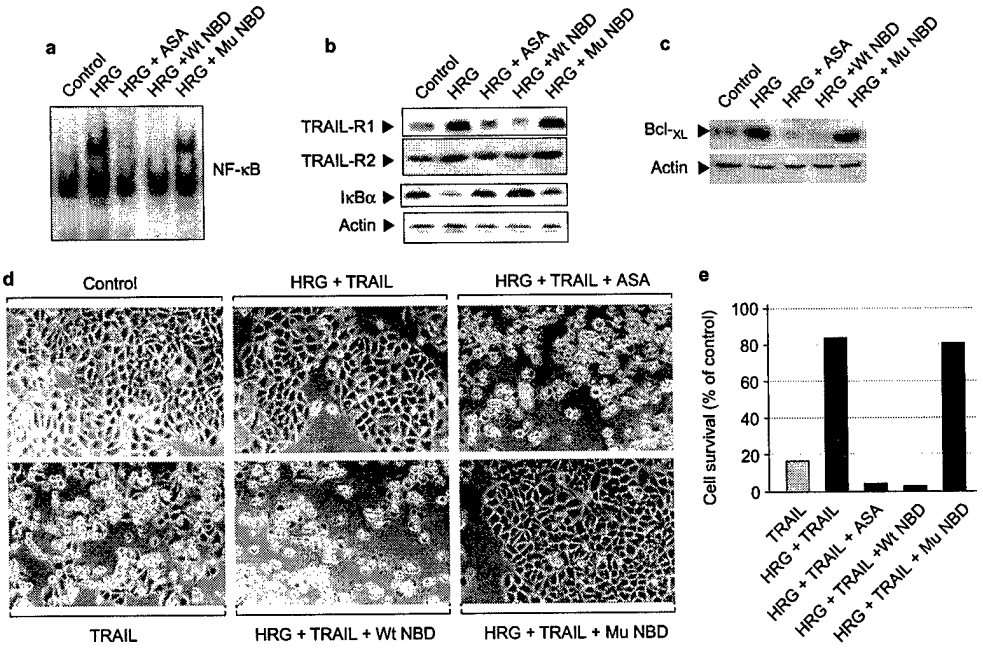

Figure 5 **Inhibition of NF-κB by blocking activation of the IKK complex sensitizes tumour cells to TRAIL. a**, EMSA of NF-κB DNA-binding activity in nuclear extracts of MCF-7 cells exposed to recombinant heregulin β1 (HRG) in the absence or presence of either aspirin (ASA; 3 mM), a cell-permeable peptide spanning the IKKβ NEMO-binding domain (wild-type (Wt NBD) or mutant (Mu NBD); 250 μM). Untreated MCF-7 cells were used as controls (Control). **b, c**, Immunoblot analyses of TRAIL-R1, TRAIL-R2, IκBα and Bcl-x_L protein expression in MCF-7 cells after exposure to HRG for 12 h (in the absence or presence of either ASA, Wt NBD or Mu NBD). Untreated MCF-7 cells served as controls. **d, e**, Untreated or HRG-treated MCF-7 cells were exposed to TRAIL (100 ng ml^{-1} + enhancer antibody 2 mg ml^{-1}) in the absence or presence of either ASA (3 mM), Wt NBD (250 μM) or Mu NBD (250 μM) for 24 h. Representative photomicrographs illustrating the survival/apoptosis of MCF-7 cells in each group are shown in **d**. Data in **e** represent the percentage survival (viable/[apoptotic + viable]) in each cell population (mean) from three independent experiments.

Discussion

NF-κB has apparently conflicting roles in the regulation of cell survival in several well-defined physiological systems and pathological states[25–39]. Targeted disruption of the RelA subunit of NF-κB results in massive hepatic apoptosis and the embryonic death of mice[26]. RelA deficiency or NF-κB inhibition by phosphorylation mutants of IκBα sensitizes cells to TNF-α-induced death[27–30]. Activation of NF-κB by co-stimulation of lymphocytes mediates cell survival and clonal proliferation, and inhibition of NF-κB by IκB mutants promotes activation-induced apoptosis of T cells, and loss of $CD8^+$ T cells in the thymus[31].

In contrast to its demonstrated protective role in these studies, NF-κB can adopt a pro-apoptotic function in other circumstances. Constitutive activation of NF-κB in mouse embryos through targeted disruption of IκBα results in a lethal phenotype manifesting thymic and splenic atrophy[36], and high levels of the c-Rel subunit of NF-κB are observed during apoptosis in the developing avian embryo[37]. NF-κB has also been reported to be essential in p53-mediated apoptosis[38]. NF-κB exhibits contrasting effects on neuronal cell survival; while it protects neurons from β-amyloid-induced death, it promotes cell death in cerebral ischaemic and neurodegenerative disorders[39]. Activation of NF-κB by ischaemic or stress-induced signals, such as hypoxia or DNA damage, may be protective in some situations and detrimental in others. These observations raise a fundamental issue of how NF-κB can have divergent effects on cell survival depending on the cell type and the specific activating signal.

Here we have shown that NF-κB induces the expression of both death receptors (TRAIL-R1,TRAIL-R2) and survival genes such as Bcl-x_L; however, the κB motifs in pro- or anti-apoptotic genes seem to exhibit selective affinity for activation by dimers composed of specific subunits of NF-κB. The varying phenotypes of knockout mice lacking individual Rel proteins reveal that the different subunits share certain functions, but also perform unique roles that cannot be complemented and may even be opposed by other family members. As κB sites on certain survival or pro-apoptotic genes exhibit specific preferences for RelA and c-Rel, the balance between different NF-κB dimers may determine the susceptibility of cells to diverse stressful stimuli that activate NF-κB.

Although our results suggest that subunit-specific regulation of death-modulating genes provides a mechanism that may underlie the seemingly paradoxical effects of NF-κB on cell survival, it is also conceivable that dimers composed of either subunit could have different effects depending on the cell type and the circumstances or duration of activation. For example, RelA seems able to stimulate expression of Fas/CD95 (ref. 48), and c-Rel can induce expression of genes such as inducible nitric oxide synthase (iNOS), interleukin-2 or Bfl-1/A1 (refs 33, 34), which may serve anti-apoptotic functions. In situations where activity of a particular subunit is deregulated, it may also adopt a promiscuous ability to induce 'death' or 'survival' genes that are not the normal transcriptional targets. As such, the final cellular response to apoptotic signals may be determined by the relative activity of different dimers comprising specific subunits, as well as by the duration and level of activity of the particular dimers involved.

Identifying approaches that sensitize cancer cells to apoptosis while concurrently protecting normal tissues might improve the

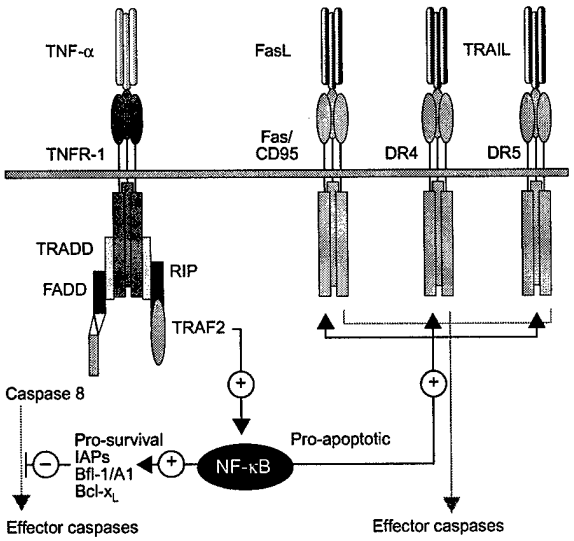

Figure 6 **Representation of the molecular determinants of the contrasting effects of NF-κB on cell survival.** TNF-induced aggregation of the death domains of TNFR1 enables recruitment of the adapter protein TRADD (TNFR1-associated death domain). The death domain of TRADD recruits FADD (Fas-associating protein with death domain)/Mort1 which, in turn, binds and activates caspase-8, the proximal member of a cascade of effector caspases that execute cell death. The TNFR1–TRADD complex also recruits proteins (TRAF2 and RIP) which signal the activation of NF-κB. Activation of NF-κB protects cells against TNF-α- or TRAIL-induced death through induction of pro-survival genes, such as members of the IAP family (c-IAP1, c-IAP2, XIAP) or the Bcl-2 homologues, Bfl-1/A-1 and Bcl-x_L. NF-κB may also function as a pro-death factor by inducing expression of death receptors (CD95/Fas,TRAIL-R1/DR4, TRAIL-R2/DR5) which trigger caspase activation and apoptosis.

therapeutic ratio of anticancer agents. Although the activation of TRAIL-R1/TRAIL-R2 signalling by TRAIL offers a potential mechanism of inducing apoptosis in tumours that resist conventional genotoxic therapy, the therapeutic ratio of this approach depends on the differential basal expression of death or decoy receptors and pro-survival proteins in tumour cells and normal tissues[20].

Our studies indicate that the composition and activity of NF-κB in tumour cells is a key determinant of the expression of TRAIL receptors or survival proteins and their susceptibility to apoptosis after ligation with TRAIL. Our data also indicate that TRAIL can synergize with genotoxic agents to eliminate p53-deficient or Bcl-2-overexpressing tumour cells that are otherwise resistant to DNA-damage-induced apoptosis. However, endogenous or cytokine-induced activation of the RelA subunit induces Bcl-x_L and protects tumour cells from TRAIL. Most significantly, our findings indicate that inhibiting NF-κB after the ligation of death receptors can reduce Bcl-x_L expression and sensitize tumour cells to TRAIL-induced apoptosis. The identified roles of NF-κB in death receptor expression and signalling may aid the rational design of regimens using TRAIL to eliminate tumour cells while sparing normal tissues. □

Methods

Cell lines and cell culture.

RelA$^{+/+}$ and RelA$^{-/-}$ mouse fibroblasts[26] (A. A. Beg, Columbia Univ., USA), c-Rel$^{-/-}$ mouse fibroblasts[40] (S. Gerondakis, WEHI, Australia), p53$^{-/-}$ MEFs (T. Jacks, MIT, USA), and MEFs stably transduced with a plasmid encoding IκBαM (pLIκBαMSN) or the empty control vector (pLXSN)[28] (D. R. Green, La Jolla Institute of Allergy and Immunology, USA) were maintained in high-glucose DMEM (Life technologies, Inc.) supplemented with 10% fetal calf serum (FCS), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C and 5% CO_2.

Genes encoding c-Rel (CCR), Δc-Rel (CCR-H; a c-Rel truncation mutant lacking the C-terminal transactivation domain owing to a stop codon at the unique *Hin*cII site of c-Rel), or RelA were conditionally expressed in HeLa (HtTA-1) cells using a tetracycline-regulated system[33,41]. HtTA-1 cells, which stably express a fusion protein comprising the *E. coli* tetracycline repressor and the activation domain of the herpes simplex virus VP16 protein (tTA), were a gift from H. Bujard (Heidelberg, Germany). We transfected HtTA-1 cells with pUHD10-3–CCR (encoding chicken c-Rel complementary DNA under control of the minimal cytomegalovirus promoter and hepatamerized tetracycline operator sites of pUHD10-3) and pHMR272 (to confer resistance to hygromycin B), pUHD10-3–hygro–CCR-H (encoding Δc-Rel), or pUHD10-3–hygro–RelA using a modified calcium phosphate procedure. Cell clones stably transduced with c-Rel, Δc-Rel or RelA were selected in hygromycin B (225 U ml^{-1}; Calbiochem) and maintained in DMEM supplemented with 10% FCS, 125 μg ml^{-1} G418 (Gibco), 225 U ml^{-1} hygromycin B (Calbiochem), 2 μg ml^{-1} tetracycline (Sigma), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C and 5% CO_2. We induced gene expression by washing the cells and transferring them to tetracycline-free medium for 48–72 h.

The parental HCT116 human colon adenocarcinoma cell line, containing wild-type p53 (*p53*$^{+/+}$), and a p53-deficient derivative (*p53*$^{-/-}$), created by homozygous deletion through homologous recombination (B. Vogelstein, Johns Hopkins Oncology Center, USA), were cultured as described[42]. We isolated primary B lymphocytes from splenocytes obtained from wild-type, Bcl-2-overexpressing (TgN(Bcl-2)36Wehi)[43] (Jackson Laboratories) and c-Rel$^{-/-}$[40] mice. *In vitro* activation of mouse B lymphocytes was performed by anti-CD40 monoclonal antibody (10 μg ml^{-1}, clone HM40-3; Pharmingen) and goat anti-mouse IgG (H+L, 10 μg ml^{-1}; Jackson ImmunoResearch). HL-60 cells transfected with an expression vector encoding Bcl-x_L (HL-60-Bcl-x_L) or control vector (HL-60-Control) (K. Bhalla, Emory University, USA) were cultured as described[49]. We inhibited caspase-3 in HL-60 cells by pre-treating the cells with Ac-DEVD-CHO (300 μM; Bachem) for 30-min before administering TRAIL.

MCF-7 human breast cancer cells were maintained in RPMI medium (Life technologies) supplemented with 10% fetal calf serum (FCS), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37 °C and 5% CO_2. MCF-7 cells were cultured in the presence or absence of recombinant heregulin β1 (100 ng ml^{-1}; Neomarkers, Fremont, CA), as indicated.

Exposure to ligands of the TNF family.

We incubated cells in either 10–100 ng ml^{-1} recombinant human TRAIL with enhancer antibody (2 μg ml^{-1}; Alexis Corporation), or 100 ng ml^{-1} rh TNF-α (R & D Systems) for 24 h at 37 °C.

Exposure to inhibitors of the IKK complex.

We inhibited the IKKβ–NEMO interaction and HRG β1-induced NF-κB activation by incubating MCF-7 cells with a 250 μM concentration of a cell-permeable peptide spanning the IKKβ NBD. The sequence of the wild-type peptide indicating the Antennapedia homeodomain (lower case) and the IKKβ (upper case) segments and the mutant peptide with the positions of the W to A mutations (underlined) are as follows[46]: wild type, drqikiwfqnrrmkwkkTALDWSWLQTE; mutant, drqikiwfqnrrmkwkkTALDASALQTE. Both peptides (Genemed Synthesis, San Francisco) were supplied as a 20 mM solution in dimethyl sulphoxide (DMSO). Results for DMSO controls were not different from controls using no peptide. We treated MCF-7 cells with 3 mM acetyl salicylic acid (aspirin; Sigma), obtained from a 1.0 M stock solution prepared in 0.05 M Tris-HCl.

Exposure to ionizing radiation.

Ionizing radiation (IR) (500 cGy) was delivered with a ^{137}Cs dual source γ-cell irradiator.

Expression vectors and cell transfections.

RelA–/– or c-Rel–/– MEFs were plated at ~50% confluence 16–24 h before serum-free lipofectin-mediated transfection (12–16 h) with either empty pCEP4 vector or pCEP4-KILLER/DR5 (ref. 10; W. El-Deiry, HHMI, Univ. Pennsylvania). Transiently transfected cell populations were assessed for apoptosis 48 h later, and then selected with 0.5 mg ml^{-1} hygromycin B for another 14 d before examination after staining with crystal violet.

Electrophoretic mobility shift assays.

Nuclear extracts were prepared as described[50]. Double-stranded oligonucleotides containing either a consensus binding site for c-Rel (5′-GGG GAC TTT CCC-3′) (Santa Cruz Biotechnology) were 5′ end-labelled using polynucleotide kinase and [γ-^{32}P]dATP. Nuclear extracts (2.5–5 μg) were incubated with ~1 μl of labelled oligonucleotide (20,000 c.p.m.) in 20 μl of incubation buffer (10 mM Tris-HCl, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 2% glycerol, 1–2 μg of poly dI-dC) for 20 min at 25 °C. The specificity of NF-κB DNA-binding activity was confirmed by competition with excess cold wild-type or mutant oligonucleotide or supershift with either a rabbit polyclonal antibody against c-Rel (Ab-3)[41] or p65 (RelA) (sc-109, Santa Cruz Biotechnology). DNA–protein complexes were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels and analysed by autoradiography.

Reporter assays.

We co-transfected CD40-activated B cells with HIV1-LTR-CAT reporter, RelA expression vector (pGD RelA) (using reporter and activator DNA in a 1:2 ratio) and a β-galactosidase expression vector (poN260). Transfected cells were irradiated (5 Gy) or left untreated, and assessed 6 h later for HIV-CAT expression using thin layer chromatography and a PhosphorImager (Molecular Dynamics)[50]. HIV-CAT activity was expressed as percentage conversion and normalized to β-galactosidase activity.

Immunoblot assays.

Cell lysates were prepared as described[50], and 50–100 μg of protein were resolved by SDS–PAGE, transferred onto Immobilon-P PVDF membrane (Millipore), and probed with appropriate dilutions of the following primary antibodies: anti-c-Rel (Ab-3)[41]; anti-IκBα (C-12), anti-p65 (RelA)(sc109), anti-Bcl-x_L (S-18), anti-Bcl-2 (100) and anti-Actin (C-11) (Santa Cruz Biotechnology); rabbit polyclonal anti-TRAIL-R1 (AHR5012; Biosource International) or anti-TRAIL-R2 (AHR5022; Biosource International). We visualized immunoreactive protein complexes by enhanced chemiluminescence (Amersham).

RNA extraction and northern blot hybridization.

Total RNA was extracted using Trizol (Life Technologies). RNA samples (20 μg) were analysed in 1.2% agarose–formaldehyde gels, transferred onto Zeta probe membranes (Biorad), and ultraviolet crosslinked with a Stratalinker (Stratagene). The membranes were hybridized to the following ^{32}P-labelled probes: (1) human TRAIL-R1/DR4 cDNA (Alexis Corporation); (2) a partial length cDNA representing a 364-bp *Eco*R1 fragment of mouse KILLER/DR5 (W. El-Deiry, HHMI, Univ. Pennsylvania); (3) human TRAIL-R2/DR5/KILLER cDNA (Alexis); and (4) β-actin. We washed membranes in SSPE/0.1% SDS at 65 °C, and visualized them by autoradiography.

Flow cytometry.

HtTA cells induced to express either c-Rel (CCR43) or Δc-Rel (CCR-H5) for 48 h were irradiated (10 Gy) or left untreated. After 12h, cells were collected with trypsin (0.25% at 37 °C for 5 min), washed twice in PBS at 4 °C and FACS buffer (PBS, 1% BSA, sodium azide 0.05%), and stained with goat anti-human TRAIL-R2 polyclonal antibody (AB1687; Chemicon International) for 30 min at 4 °C. Cells were washed twice with FACS buffer, exposed to PE-conjugated donkey anti-goat IgG for another 30 min at 4 °C (Sigma), and analysed by a FACScan flow cytometer (Becton Dickinson). Cells treated with secondary antibody alone were used as unstained negative controls.

Confocal microscopy.

HtTA cells were seeded onto eight-well chamber slides (Nunc, Naperville, IL) and induced to express either c-Rel (CCR43) or Δc-Rel (CCR-H5) for 48 h before fixation in 2% paraformaldehyde for 5 min. We permeabilized the cells with 0.1% saponin in PBS containing 10% human AB serum for 10 min, and then incubated them with goat anti-human TRAIL-R2 polyclonal antibody (AB1687; Chemicon International)(1:300 in PBS containing 1% human AB serum) at 4 °C for 1 h. After washing with PBS, cells were incubated with FITC-conjugated donkey anti-goat IgG (H+L) secondary antibodies (Molecular Probes, Eugene, OR) at 4 °C for 45 min. Cells were counterstained with propidium iodide and examined using a Zeiss Axiophot microscope with a confocal attachment and a digital camera.

Analysis of cell death.

Cells were assessed for morphological features of apoptosis using phase-contrast microscopy. We quantified cell survival at the indicated intervals by trypan blue dye exclusion. The cell viability was measured by scoring at least 200 cells in each group, and the average per cent viability was calculated from three different experiments.

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Appendix 3: Unpublished Data: (Page 33-34)

Figure

Sensitization of breast cancer cells to hypoxia-induced apoptosis by inhibition of NF-κB.
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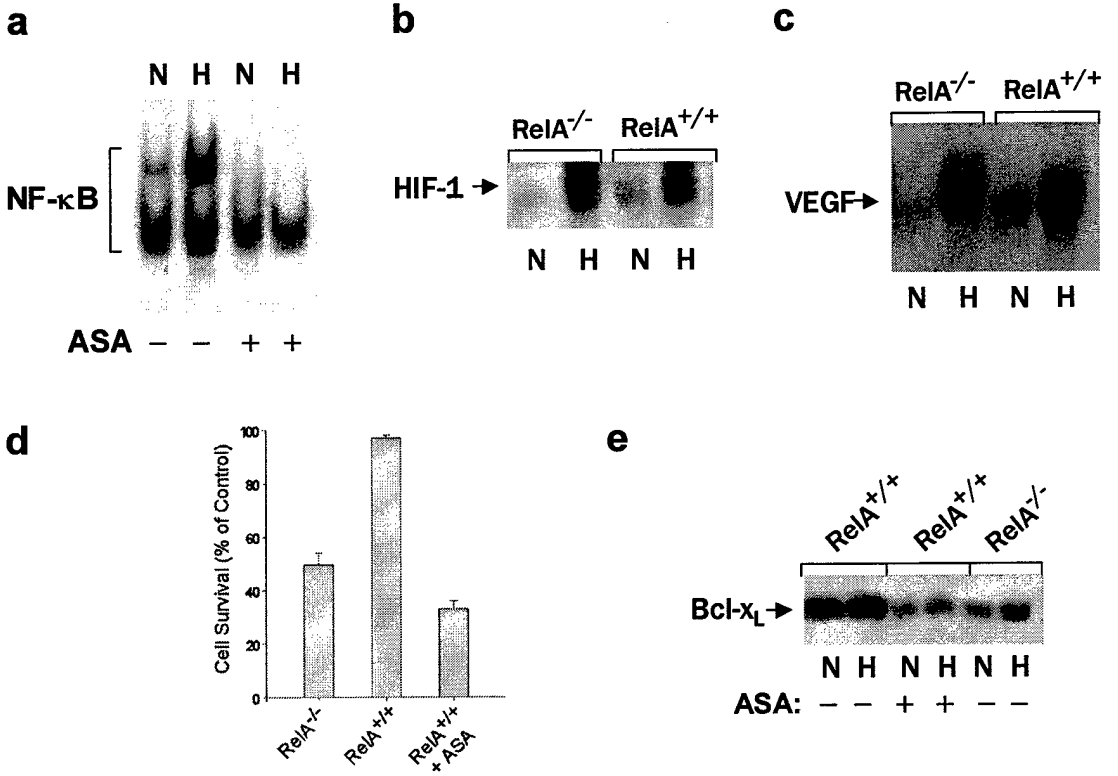
Inhibition of NF-κB sensitizes breast cancer cells to hypoxia-induced apoptosis.

Figure Legend

NF-κB/RelA induces expression of Bcl-x_L and protects cells from hypoxia-induced apoptosis. a. Electrophoretic mobility shift assay of NF-κB DNA binding activity in 3T3 cells in the presence or absence of aspirin (ASA, 1 mM). **b.** Immunoblot assay of HIF-1a expression in $RelA^{+/+}$ and $RelA^{-/-}$ fibroblasts in either normoxia (20% O_2)(N) or hypoxia (0.1% O_2)(H). **c.** Northern blot analysis of VEGF mRNA expression in $RelA^{+/+}$ and $RelA^{-/-}$ fibroblasts in either normoxia (20% O_2)(N) or hypoxia (0.1% O_2)(H). **d.** Survival of $RelA^{+/+}$ and $RelA^{-/-}$ fibroblasts following exposure to 0.1% O_2 for 48h. **e.** Immunoblot analysis of Bcl-x_L expression in $RelA^{+/+}$ and $RelA^{-/-}$ fibroblasts in either normoxia (20% O_2)(N) or hypoxia (0.1% O_2)(H), in the presence or absence of ASA (1 mM).

Appendix 3: Figure



Appendix 4: Reprint of publication: (Page Numbers 35-40)

Ravi, R. and Bedi, A. Sensitization of Tumor Cells to Apo2L/TRAIL-induced Apoptosis by Inhibition of Casein Kinase II. *Cancer Research 62*: 4180-4185, 2002.

Sensitization of Tumor Cells to Apo2 Ligand/TRAIL-induced Apoptosis by Inhibition of Casein Kinase II[1]

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Abstract

Tumor-cell death can be triggered by engagement of specific death receptors with Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL). Apo2L/TRAIL-induced apoptosis involves caspase-8-mediated cleavage of BID. The active truncated form of BID (tBID) triggers the mitochondrial activation of caspase-9 by inducing the activation of BAK or BAX. Although a broad spectrum of human cancer cell lines express death receptors for Apo2L/TRAIL, many remain resistant to TRAIL/Apo2L-induced death. A variety of human cancers exhibit increased activity of casein kinase II (CK2). Here we demonstrate that CK2 is at the nexus of two signaling pathways that protect tumor cells from Apo2L/TRAIL-induced apoptosis. We find that CK2 inhibits Apo2L/TRAIL-induced caspase-8-mediated cleavage of BID, thereby reducing the formation of tBID. In addition, CK2 promotes nuclear factor κB (NF-κB)-mediated expression of Bcl-x_L, which sequesters tBID and curtails its ability to activate BAX. Tumor cells with constitutive activation of CK2 exhibit a high Bcl-x_L/tBID ratio and fail to activate caspase-9 or undergo apoptosis in response to Apo2L/TRAIL. Conversely, reduction of the Bcl-x_L/tBID ratio by inhibition of CK2 renders such cancer cells sensitive to Apo2L/TRAIL-induced activation of caspase-9 and apoptosis. Using isogenic cancer cell lines that differ only in the presence or absence of either the *p53* tumor suppressor or the *BAX* gene, we show that the enhancement of Apo2L/TRAIL-induced tumor-cell death by CK2 inhibitors requires BAX, but not p53. The identification of CK2 as a key survival signal that protects tumor cells from death-receptor-induced apoptosis could aid the design of Apo2L/TRAIL-based combination regimens for treatment of diverse cancers.

Introduction

Genetic aberrations that render cells incapable of executing apoptosis not only promote tumorigenesis, but also underlie the observed resistance of human cancers to anticancer agents. Unraveling mechanisms to unleash the apoptotic program in tumor cells could aid the design of effective therapeutic interventions against resistant cancers. Tumor-cell death can be triggered by engagement of specific death receptors belonging to the TNF-receptor[3] gene superfamily with the "death ligand," Apo2L/TRAIL (1, 2). TRAIL/Apo2L induces apoptosis of many cancer cell lines *in vitro*, and its tumoricidal activity and safety *in vivo* has been confirmed in preclinical animal models of human cancer xenografts (2). Although most human cancer cell lines express death receptors for Apo2L/TRAIL, many remain resistant to TRAIL/Apo2L-induced death (2). The identification of key survival signals responsible for protecting tumor cells from death-receptor-induced apoptosis could aid the design of Apo2L/TRAIL-based combination regimens for treatment of such cancers.

The death receptors for TRAIL/Apo2L, TRAIL-R1 (DR4), and TRAIL-R2 (DR5, KILLER) are type I transmembrane proteins containing cytoplasmic sequences, termed "death domains," that recruit and cross-activate the initiator procaspase-8 (1). Caspase-8 cleaves and activates BID, a "BH-3 domain only" prodeath member of the Bcl-2 family (3, 4). The active truncated form of BID (tBID) triggers the mitochondrial activation of caspase-9 by inducing the homooligomerization and allosteric activation of BAK or BAX, two multidomain proapoptotic members of the Bcl-2 family (5). However, death-receptor-induced activation of caspase-9 is inhibited by Bcl-x_L, an NF-κB-inducible Bcl-2 family member that sequesters tBID and curtails its ability to activate BAX (6–8). Therefore, molecules that govern the relative balance between tBID and Bcl-x_L may be key determinant(s) of the sensitivity of tumor cells to Apo2L/TRAIL-induced death.

Protein kinase CK2 is an evolutionarily conserved holoenzyme composed of two catalytic α (and/or a') subunits and two regulatory β subunits (9). CK2 is increased in response to diverse growth stimuli, and its activity is aberrantly elevated in diverse tumor types including breast carcinomas, colorectal carcinomas, squamous cell carcinomas and adenocarcinomas of the lung, squamous cell carcinomas of the head and neck, prostate carcinomas, ovarian carcinomas, and melanomas (9). Adult transgenic mice expressing CK2α in lymphocytes display a stochastic propensity to develop lymphoma (10), and overexpression of CK2α in the mammary gland results in breast tumors (11). In addition to its demonstrated role in oncogenesis, we now report that CK2 is at the nexus of two survival signaling pathways that protect tumor cells from Apo2L/TRAIL-induced apoptosis. We find that CK2 inhibits Apo2L/TRAIL-induced caspase-8-mediated cleavage of BID. In addition to preventing the formation of tBID, our studies demonstrate that CK2 promotes NF-κB-mediated expression of Bcl-x_L. We show that cancer cells with constitutive activation of CK2 exhibit a high Bcl-x_L/tBID ratio and fail to activate caspase-9 or undergo apoptosis in response to Apo2L/TRAIL. Conversely, reduction of the Bcl-x_L/tBID ratio by inhibition of CK2 with DRB (12), the natural plant flavonoid, apigenin (13), or the anthraquinone derivative, emodin (14), renders such cancer cells sensitive to Apo2L/TRAIL-induced activation of caspase-9 and apoptosis. Using isogenic cancer cell lines that differ only in the presence or absence of either the *p53* tumor suppressor gene or the *BAX* gene (15, 16), we further demonstrate that TRAIL/Apo2L-induced death of $p53^{+/+}$- or $p53^{-/-}$- BAX-proficient, but not BAX-deficient, cancer cells is augmented by inhibition of CK2. These observations indicate that CK2 inhibitors can

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[3] The abbreviations used are: TNF, tumor necrosis factor; Apo2L, Apo2 ligand; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; NF-κB, nuclear factor-κB; CK2, casein kinase II; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; GST, glutathione S-transferase; IKK, inhibitor of κB kinase; tBID, truncated form of BID; ASA, acetyl salicylic acid; CAT, chloramphenicol acetyltransferase; IκBα, inhibitor of κB; FLIP, FLICE-inhibitory protein.

augment Apo2L/TRAIL-induced tumor-cell death by elevating the tBID/Bcl-x_L ratio and promoting the activation of BAX.

Materials and Methods

Cell Lines and Cell Culture. The HCT116 human colon adenocarcinoma cell line containing wild-type *p53* and one intact *BAX* allele ($p53^{+/+}BAX^{+/-}$) and isogenic p53-deficient ($p53^{-/-}$) or BAX-deficient ($BAX^{-/-}$) derivatives of HCT116 cells generated by disruption of either *p53* or *BAX* alleles by gene targeting have been described previously (15, 16). HCT116 cells of each genotype ($p53^{+/+}$,$BAX^{+/-}$; $p53^{-/-}$; $BAX^{-/-}$) were provided by Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). Isogenic HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml). The Hs578 and SKBr-3 human breast cancer cell lines were cultured in DMEM (with 1 μg/ml insulin) and McCoy's 5A medium, respectively, supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Treatment with Recombinant Human TRAIL/Apo2L. Exponentially growing cells (2×10^5/well in 6-well plates) were incubated with soluble recombinant human TRAIL/Apo2L (100 ng ml^{-1}) plus enhancer antibody (2 μg ml^{-1}; Alexis, San Diego, CA) for 48 h at 37°C.

Immunoblot Assays. Cell lysates were prepared as described (7), 50–100 μg of protein were resolved by SDS-PAGE, transferred onto Immobilon-P PVDF membrane (Millipore, Bedford, MA), and probed with antibodies against caspase-8 (C-20), BID (C-20), BAX (N-20), caspase-9 (H-170), Bcl-x_L (S-18), FLIP (G-11), CK2α (C-18), and actin (C-11) from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoreactive protein complexes were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL).

CK2 Kinase Assays. The phosphotransferase activity of CK2 was measured using a Casein Kinase-2 Assay Kit (Upstate Biotechnology, Lake Placid, NY). This assay is based on phosphorylation of a CK2-specific peptide substrate using the transfer of the γ-phosphate of [γ-^{32}P]ATP by CK-2 kinase. The phosphorylated substrate was separated from the residual [γ-^{32}P]ATP using P81 phosphocellulose paper, and [^{32}P] incorporation into the substrate was measured using a scintillation counter and expressed as the calculated pmol phosphate incorporated into CK2 substrate peptide/min/200 ng of CK-2.

CK2 complexes were immunoprecipitated from whole-cell extracts (500 μg) using an antibody against CK2α (C-18; Santa Cruz Biotechnology) and subjected to a CK2 kinase assay at 30°C for 30 min in kinase buffer {100 mM Tris (pH 8.0), 100 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, 10 μCi [γ-^{32}P]GTP, 100 μM Na_3VO_4, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin} containing 200 ng of GST-IκBα fusion protein (GST-IκBα; Santa Cruz Biotechnology) as substrate (17). The specificity of the kinase reaction was confirmed by the addition of cold CK2-specific peptide substrate H-Arg-Arg-Ala-Asp-Asp-Ser-Asp-Asp-Asp-Asp-Asp-OH (Calbiochem, San Diego, CA). Recombinant CK2 (New England Biolabs) was used as a positive control. The kinase reaction was terminated by the addition of 2× SDS-PAGE sample buffer and subjected to SDS-PAGE and autoradiography.

Inhibition of CK2 was achieved by incubation of cells in graded concentrations of DRB(10 or 40 μM; Calbiochem; Ref 12), apigenin (10 or 20 μM; Sigma-Aldrich, St. Louis, MO; Ref. 13), or emodin (5, 10, or 20 μg/ml; Sigma-Aldrich; Ref. 14).

IKK Kinase Assay. IKK complexes were immunoprecipitated from whole-cell extracts (500 μg) using an antibody against IKKβ (M-280; Santa Cruz Biotechnology) and subjected to a kinase assay at 30°C for 30 min in kinase buffer [20 mM HEPES (pH 7.6), 3 mM $MgCl_2$, 10 μM ATP, 3 μCi [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM *p*-nitrophenyl phosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT] containing 500 ng of glutathione S-transferase (GST)-IκBα fusion protein (GST-IκBα; Santa Cruz Biotechnology) as substrate (18). The kinase reaction was terminated by the addition of 2× SDS-PAGE sample buffer and subjected to SDS-PAGE and autoradiography. Inhibition of IKK was achieved by incubation of cells with either ASA (3 mM) or sulindac sulfide (120 μM) from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA).

Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared as described (7). Double-stranded oligonucleotides containing a consensus binding site for NF-κB (5′-GGGGACTTTCCC-3′; Santa Cruz Biotechnology) were 5′-end labeled using polynucleotide kinase and [γ-^{32}P] dATP. Nuclear extracts (2.5–5 μg) were incubated with ~1 μl of labeled oligonucleotide (20,000 cpm) in 20 μl incubation buffer [10 mM Tris, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 2% glycerol, and 1–2 μg of poly(deoxyinosinic-deoxycytidylic acid] for 20 min at 25°C. The specificity of NF-κB DNA-binding activity was confirmed by competition with excess cold wild-type or mutant oligonucleotide or supershift with an antibody against p65/RelA (Geneka, Montreal, Canada) as described (7). DNA-protein complexes were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels and analyzed by autoradiography and densitometry (Molecular Dynamics).

Transient Transfection and NF-κB-dependent *bcl-x*-CAT Reporter Assay. The *bcl-x*-CAT reporter containing the human *bcl-x* promoter region cloned in a promoterless vector expressing CAT reporter gene (pCAT-basic) and the κB site mutant *bcl-x*-CAT plasmid with an inactivated NF-κB motif at position −232 in the *bcl-x* promoter (TTTACTGCCC; −298/+22 mκB) have been described previously (provided by Dr. Céline Gelinas, University of Medicine and Dentistry of New Jersey, NJ; Ref. 6). Hs578 cells were transiently cotransfected with 1 μg of either *bcl-x*-CAT reporter plasmid (−298/+22) or the κB site mutant (mt) plasmid (−298/+22 mκB) together with a β-galactosidase (CMV-β-gal) reporter using lipofectin (InVitrogen). Transfected cells were incubated at 37°C for 24 h in DMEM medium (supplemented with insulin) in the absence or presence of DRB (10 or 40 μM), apigenin (10 or 20 μM), or emodin (5 or 10 μg/ml). Cells were then harvested and assayed for CAT activity (normalized to β-gal activity) as described (6).

Analysis of Cell Death. Cells were assessed for morphological features of apoptosis (condensed chromatin and micronucleation) by microscopic visualization. Cell viability was assessed at the indicated intervals by trypan blue dye exclusion of harvested cells (adherent + floating in the medium). Cell survival was measured by scoring at least 500 cells in each group, and the average percentage of viability (mean ± SE) was calculated from three different experiments.

Results

CK2 Promotes NF-κB-mediated Expression of Bcl-x_L and c-FLIP. CK2 is constitutively activated in Hs578 breast cancer cells (Fig. 1*A*; Ref. 17). Exposure of Hs578 cells to the classic CK2-specific inhibitor, DRB (10 or 40 μM), resulted in dose-dependent inhibition of phosphorylation of a CK2-specific peptide substrate (RRADDSDDDDD; Fig. 1*A*). Dose-dependent inhibition of CK2 activity in Hs578 cells was also achieved by treatment with the plant flavonoid, apigenin (10 or 20 μM), or the plant anthraquinone derivative, emodin (10 or 20 μg/ml), but not by the nonsteroidal anti-inflammatory drugs, ASA or sulindac sulfide (Fig. 1*A*).

CK2 phosphorylates the PEST domain of IκBα (19). To analyze CK2-dependent phosphorylation of IκBα in Hs578 cells, CK2α was immunoprecipitated from whole-cell lysates and subjected to an *in vitro* CK2 kinase assay using a GST-IκBα fusion protein as substrate. Kinase assays demonstrated strong constitutive phosphorylation of GST-IκBα that was reduced by competition with a CK2-specific peptide substrate (Fig. 1*B*). The specific involvement of CK2 in the high basal IκBα-kinase activity was also confirmed by treatment of cells with the CK2-inhibitors, DRB, apigenin, or emodin. Incubation of Hs578 cells with DRB (40 μM), apigenin (10, 20 μM), or emodin (5, 10 μg/ml) led to a dose-dependent inhibition of GST-IκBα phosphorylation by CK2 immune complexes, but did not influence the phosphorylation of GST-IκBα by IKKβ (Fig. 1*B*). Conversely, treatment of Hs578 cells with the nonsteroidal anti-inflammatory drugs, ASA, or sulindac sulfide inhibited IKKβ-mediated GST-IκBα phosphorylation, but did not interfere with CK2-dependent phosphorylation of GST-IκBα (Fig. 1*B*).

In addition to promoting phosphorylation-induced degradation of IκBα, CK2 activates NF-κB transcriptional activity by phosphorylating RelA/p65 (20). To evaluate whether the constitutive activation of CK2 promotes NF-κB activity, nuclear extracts of Hs578 cells incubated for 16 h with graded concentrations of DRB, apigenin, or

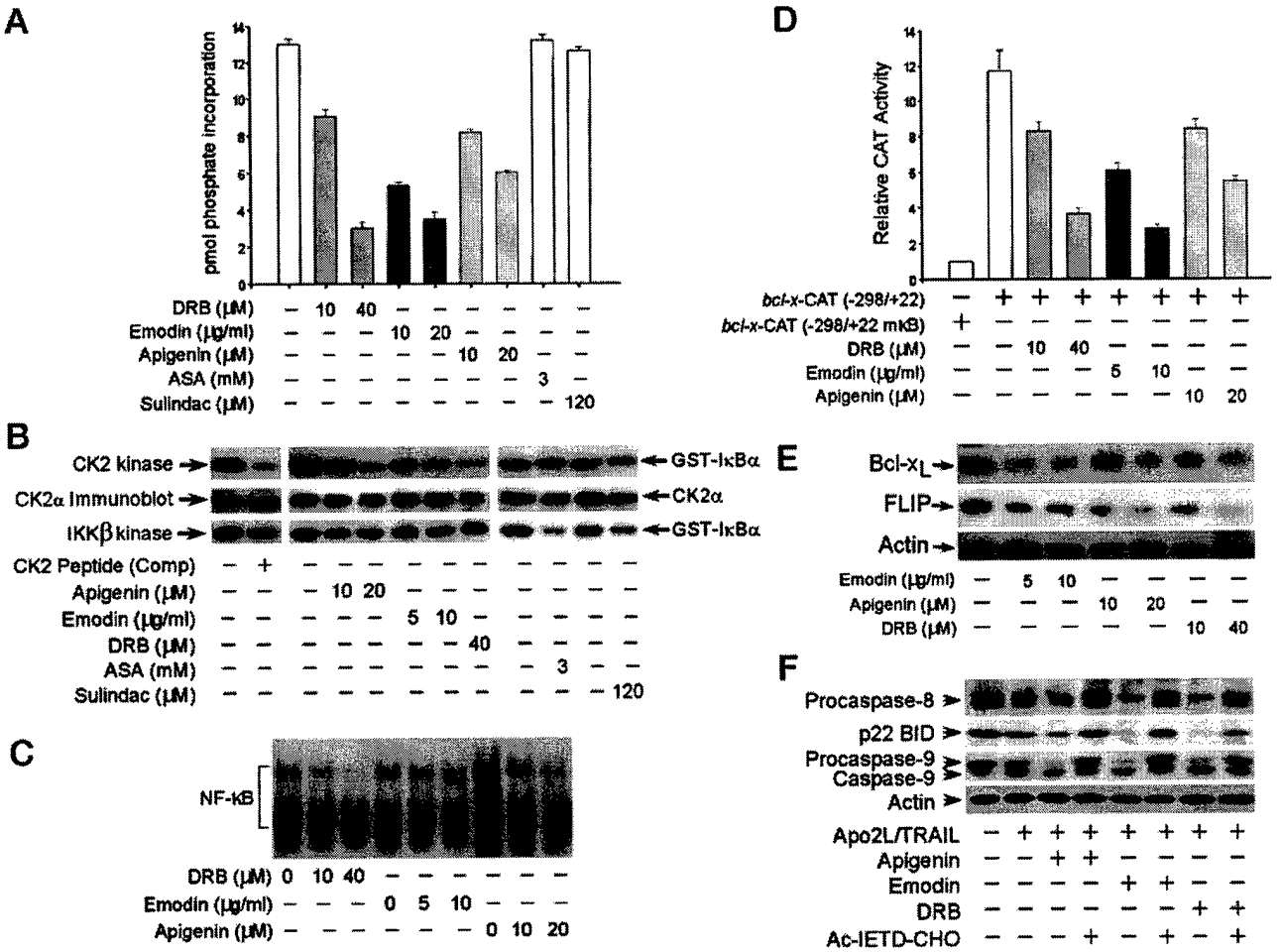

Fig. 1. CK2 inhibits the Apo2L/TRAIL-induced caspase-8-tBID-caspase-9 death signaling pathway by promoting NF-κB-mediated expression of Bcl-x_L and c-FLIP. *A*, effect of DRB, apigenin, emodin, ASA, or sulindac sulfide on CK2 kinase activity in Hs578 cells. Cells were treated with the indicated concentrations of each agent for 16 h or left untreated and harvested for measurement of the phosphotransferase activity of CK2 using a CK2-specific peptide substrate (RRADDSDDDDD). The data represent the calculated pmol phosphate incorporated into CK-2 substrate peptide/min/200 ng of CK-2 (mean of three independent experiments; *bars*, ±SE). *B*, effect of DRB, apigenin, emodin, ASA, or sulindac sulfide on CK2- or IKK-dependent phosphorylation of GST-IκBα fusion protein. Hs578 cells were treated with the indicated concentrations of each agent or left untreated for 16 h. CK2α or IKKβ were immunoprecipitated from whole-cell lysates and immune complexes were subjected to an *in vitro* kinase assay using GST-IκBα as substrate. CK2-dependent phosphorylation of GST-IκBα was confirmed by competition with a CK2-specific peptide substrate (RRADDSDDDDD). *C*, NF-κB DNA-binding activity (electrophoretic mobility shift assay) in nuclear extracts of Hs578 cells treated with the indicated concentrations of DRB, apigenin, or emodin. *D*, effect of CK2 inhibitors on NF-κB-dependent transcriptional activation of Bcl-x_L. Hs578 cells were cotransfected with either the bcl-x-CAT reporter plasmid (−298/+22) or the κB site mutant (mt) plasmid (−298/+22 mκB) and CMV-β-gal, incubated in insulin-supplemented medium with the indicated concentrations of DRB, apigenin, or emodin and then assayed for relative CAT activity (normalized to β-gal activity). The data represent the mean of three independent experiments; *bars*, ±SE. *E*, Western blot analysis of Bcl-x_L and FLIP in Hs578 cells treated with the indicated concentrations of DRB, apigenin, or emodin for 16 h. *F*, Western blot analyses of procaspase-8, BID (p22), and caspase-9 [the inactive zymogen (procaspase-9) and the active subunit resulting from its cleavage (caspase-9)] in whole-cell lysates of Hs578 cells after 24 h of treatment with DRB, apigenin, or emodin in the presence or absence of Apo2L/TRAIL (with or without the caspase inhibitor N-acetyl-Ile-Glu-Thr-Asp-CHO, Ac-IETD-CHO).

emodin were subjected to electrophoretic mobility shift assays for NF-κB DNA-binding activity. Treatment with DRB, apigenin, or emodin, led to inhibition of NF-κB DNA-binding activity (Fig. 1*C*).

The human *bcl-x* promoter contains a κB DNA site (TTTACTGCCC; 298/+22) responsible for its Rel-dependent induction (6). To determine whether CK2 promotes NF-κB-dependent transcriptional activation of Bcl-x_L, Hs578 cells were transiently cotransfected with either the *bcl-x*-CAT reporter plasmid (−298/+22) or the κB site mutant (mt) plasmid (−298/+22 mκB) and incubated in insulin-supplemented medium in the absence or presence of DRB, apigenin, or emodin. Inhibition of CK2 with DRB, apigenin, or emodin led to a dose-dependent loss of NF-κB-dependent transcriptional activation of the *bcl-x*-CAT reporter in Hs578 cells (Fig. 1*D*). Consistent with the decline of NF-κB-dependent *bcl-x* transcription by inhibition of CK2, exposure of Hs578 cells to DRB, apigenin, or emodin led to a dose-dependent reduction of Bcl-x_L protein levels in immunoblot analysis (Fig. 1*E*). Akin to Bcl-x_L, CK2 inhibitors also reduced expression of c-FLIP, an NF-κB-inducible protein that prevents death-receptor-induced activation of the initiator pro-caspase-8 (Fig. 1*E*; Ref 21). These data indicate that CK2 activation promotes NF-κB-mediated expression of Bcl-x_L and FLIP in tumor cells.

CK2 Reduces Apo2L/TRAIL-induced Formation of tBID and Promotes Its Sequestration by Bcl-x_L. Engagement of death receptors for TRAIL/Apo2L leads to recruitment and cross-activation of the initiator caspase-8, which in turn, cleaves and activates BID (3, 4). The active truncated form of BID (tBID) triggers the mitochondrial activation of caspase-9 via activation of BAK or BAX (5, 18, 22). CK2 promotes NF-κB-mediated expression of the caspase-8-inhibitor, c-FLIP (Fig. 1*E*), and phosphorylation of BID by CK2 has been

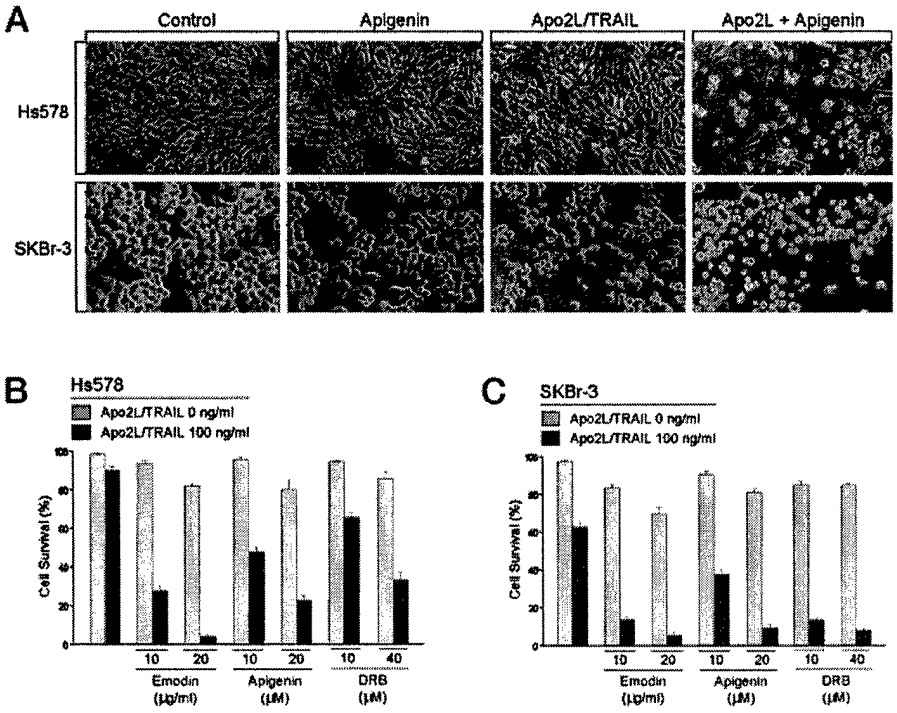

Fig. 2. Sensitization of tumor cells to Apo2L/TRAIL-induced apoptosis by inhibition of CK2. *A*, phase contrast photomicrographs of Hs578 or SKBr-3 breast cancer cells after 48 h of treatment with Apo2L/TRAIL (100 ng/ml), apigenin (20 μM), or the combination of Apo2L/TRAIL and apigenin. *B*, survival of Hs578 cells after 48 h of the indicated treatments (mean of three independent experiments; *bars*, ±SE). *C*, survival of SKBr-3 cells after 48 h of the indicated treatments (mean of three independent experiments; *bars*, ±SE).

reported to render BID resistant to caspase-8 mediated cleavage (23). In addition, CK2 promotes expression of Bcl-x_L (Fig. 1*E*), which in turn, sequesters tBID and prevents tBID-induced activation of caspase-9 (8).

To assess whether the constitutive activation of CK2 in tumor cells plays a role in regulating Apo2L/TRAIL-induced caspase-8-mediated cleavage of BID and activation of caspase-9, Hs578 cells were treated with Apo2L/TRAIL in the absence or presence of DRB, apigenin, or emodin. Immunoblot analyses showed that treatment with any of these CK2 inhibitors facilitated Apo2L/TRAIL-induced cleavage of procaspase-8, caspase-8-mediated truncation of BID, and activation of caspase-9 (Fig, 1*F*). These data indicate that elevation of the Bcl-x_L/tBID ratio by constitutive activation of CK2 in tumor cells inhibits the activation of caspase-9 in response to Apo2L/TRAIL. Conversely, reduction of the Bcl-x_L/tBID ratio by CK2 inhibitors facilitates Apo2L/TRAIL-induced activation of caspase-9 in tumor cells.

Sensitization of Tumor Cells to Apo2L/TRAIL-induced Apoptosis by Inhibition of CK2. Hs578 cells were markedly resistant to Apo2L/TRAIL, with death of only 9 ± 3% death over 48 h. To determine whether inhibition of CK2 could sensitize cancer cells to Apo2L/TRAIL-induced death, Hs578 cells were treated for 48 h with graded concentrations of DRB, apigenin, or emodin in the presence or absence of Apo2L/TRAIL (100 ng/ml). DRB, apigenin, or emodin alone induced only limited cell death at the maximum concentrations used (Fig. 2, *A* and *B*). However, treatment with any of these CK2 inhibitors led to a dose-dependent increase in Apo2L/TRAIL-induced death of Hs578 cells (Fig. 2, *A* and *B*). Similarly, inhibition of CK2 with DRB, apigenin, or emodin also led to dose-dependent sensitization of HER2/neu-overexpressing SKBr-3 breast cancer cells to Apo2L/TRAIL-induced apoptosis (Fig. 2, *A* and *C*).

CK2 Inhibitors Augment Apo2L/TRAIL-induced Tumor-Cell Death via Activation of BAX. The active truncated form of BID (tBID) triggers the mitochondrial activation of caspase-9 in tumor cells by inducing the homooligomerization and allosteric activation of BAX (18, 22). The sequestration of tBID by Bcl-x_L counteracts the mitochondrial activation of caspase-9 (8, 18). By increasing the ratio of tBID to Bcl-x_L, CK2 inhibitors may facilitate Apo2L/TRAIL-induced activation of caspase-9 via BAX. To determine whether CK2 inhibitors augment Apo2L/TRAIL-induced apoptosis of tumor cells via activation of BAX, we studied isogenic derivatives of HCT116 colon cancer cells that differ only in the presence or absence of the *BAX* gene (16). Ninety-four percent of HCT116 cells have an intact *BAX* allele ($BAX^{+/-}$) and express functional BAX protein. BAX-deficient HCT116 cells ($BAX^{-/-}$) were generated by targeted inactivation of the wild-type *BAX* allele in a BAX heterozygote (16). $BAX^{+/-}$ or $BAX^{-/-}$ HCT116 cells were treated for 48 h with graded concentrations of either apigenin (10, 20 μM) or emodin (10, 20 μg/ml) in the presence or absence of Apo2L/TRAIL (100 ng/ml). Inhibition of CK2 with either apigenin or emodin led to a dose-dependent augmentation of TRAIL/Apo2L-induced death of BAX-proficient HCT116 cells ($BAX^{+/-}$; Fig. 3, *A–C*). In contrast, BAX-deficient HCT116 cells ($BAX^{-/-}$) remained resistant to TRAIL/Apo2L-induced death even in the presence of the highest tested concentrations of either apigenin or emodin (Fig. 3, *A–C*).

Enhancement of Apo2L/TRAIL-induced Tumor-Cell Death by CK2 Inhibitors Is Independent of p53. CK2-mediated phosphorylation of p53 has been reported to modulate p53-dependent transcription (24). CK2α transgenic mice that are deficient in p53 develop thymic lymphomas at a markedly accelerated rate when compared with p53-deficient mice lacking the transgene (25). To determine whether the augmentation of Apo2L/TRAIL-induced apoptosis by CK2 inhibitors requires p53, we studied isogenic derivatives of HCT116 colorectal cancer cells that differ only in the presence or absence of the *p53* gene. HCT116 cells have wild-type *p53* ($p53^{+/+}$) and express intact functional p53 protein (15). Isogenic p53-deficient derivatives of HCT116 cells were generated by targeted inactivation of both *p53* alleles ($p53^{-/-}$; Ref. 15). $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells were treated for 48 h with graded concentrations of either apigenin (10, 20 μM) or emodin (10, 20 μg/ml) in the presence or absence of Apo2L/TRAIL (100 ng/ml). In contrast to isogenic

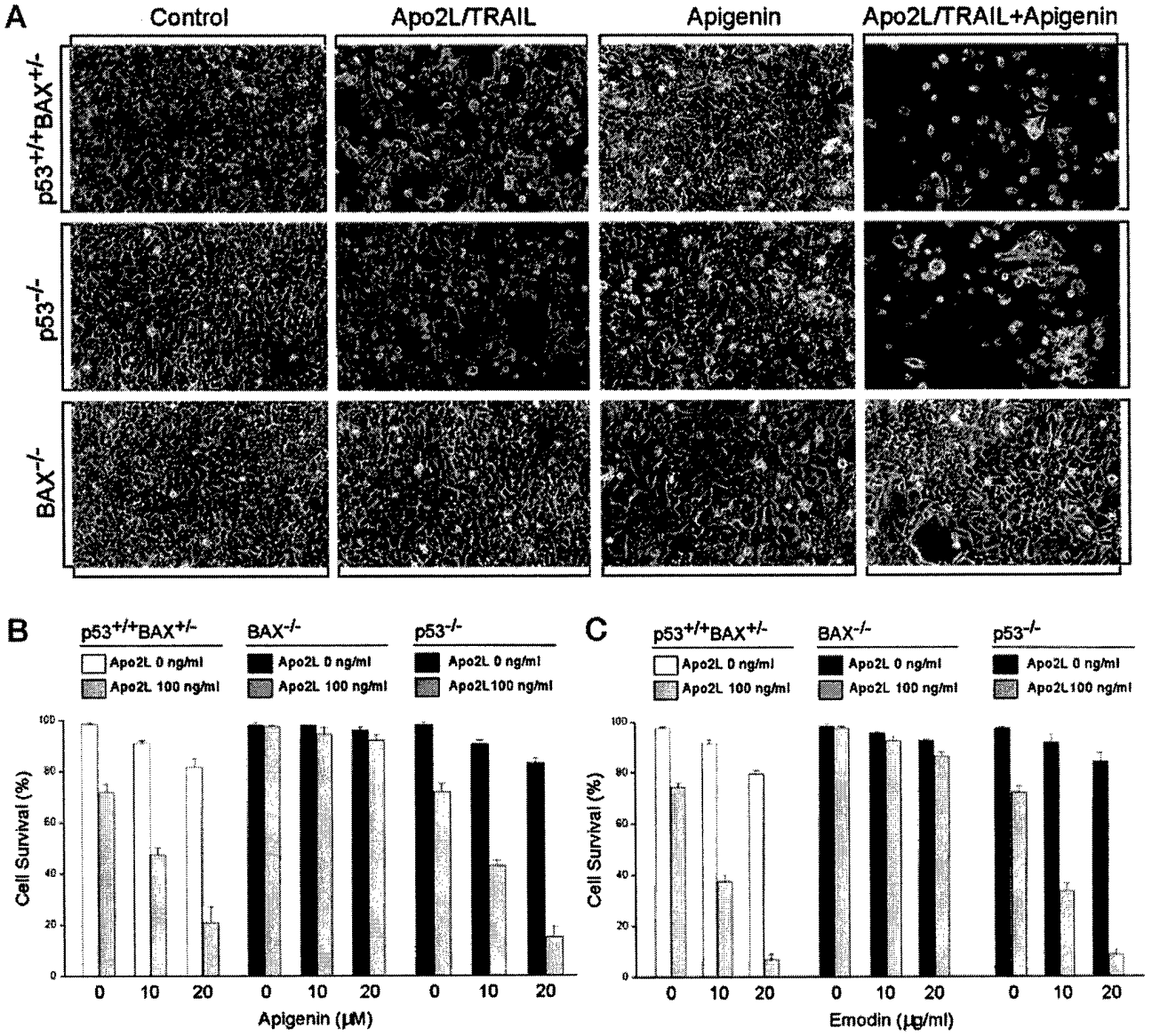

Fig. 3. TRAIL/Apo2L-induced death of $p53^{+/+}$ or $p53^{-/-}$ BAX-proficient, but not BAX-deficient, cancer cells is augmented by inhibition of CK2. *A*, phase contrast photomicrographs of $p53^{+/+}BAX^{+/-}$, $p53^{-/-}$, or $BAX^{-/-}$ HCT116 colorectal cancer cells after 48 h of treatment with TRAIL/Apo2L (100 ng/ml), apigenin (20 μM), or both TRAIL/Apo2L and apigenin. *B*, survival of $p53^{+/+}BAX^{+/-}$, $BAX^{-/-}$, or $p53^{-/-}$ HCT116 cells after 48 h of treatment with graded concentrations of apigenin (0, 10, or 20 μM) in the presence or absence of TRAIL/Apo2L (100 ng/ml); (mean of three independent experiments; *bars*, ±SE). *C*, survival of $p53^{+/+}BAX^{+/-}$, $BAX^{-/-}$, or $p53^{-/-}$ HCT116 cells after 48 h of treatment with graded concentrations of emodin (0, 10, or 20 μg/ml) in the presence or absence of TRAIL/Apo2L (100 ng/ml); (mean of three independent experiments; *bars*, ±SE).

$BAX^{-/-}$ HCT116 cells, $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells exhibited equivalent sensitivity to induction of apoptosis by either TRAIL/Apo2L or each of the CK2 inhibitors (Fig. 3, *A–C*). Exposure to either apigenin or emodin augmented the sensitivity of both $p53^{-/-}$ and $p53^{+/+}$ HCT116 cells to TRAIL/Apo2L in a dose-dependent manner (Fig. 3, *A–C*). These data indicate that the enhancement of Apo2L/TRAIL-induced tumor-cell death by CK2 inhibitors is independent of p53.

Discussion

Antibody or ligand-mediated engagement of death receptors for Apo2L/TRAIL offers an attractive strategy for inducing apoptosis of tumor cells. However, cancer cell lines exhibit a wide heterogeneity in their sensitivity to Apo2L/TRAIL-induced apoptosis, and several tumor cell lines remain resistant to Apo2L/TRAIL, even though they express death receptors, TRAIL-R1/DR4, and TRAIL-R2/DR5 (2). The efficacy of Apo2L/TRAIL against such cancers may be improved by inhibition of the critical molecule(s) that interfere with Apo2L/TRAIL-induced death signaling. Apo2L/TRAIL-induced tumor-cell death involves caspase-8-mediated cleavage of BID, tBID-mediated activation of BAX, and BAX-mediated mitochondrial activation of caspase-9 (18, 22). The results of our study indicate that CK2 is at the nexus of two survival signaling pathways that play an instrumental role in protecting tumor cells from Apo2L/TRAIL-induced apoptosis. We find that constitutive activation of CK2 in tumor cells inhibits caspase-8-mediated formation of tBID in response to Apo2L/TRAIL.

In addition, CK2 promotes NF-κB-mediated expression of Bcl-x_L, which in turn, sequesters tBID and curtails its ability to activate BAX. Consistent with the high expression of Bcl-x_L and reduced formation of tBID, we find that tumor cells with constitutively high CK2 activity fail to activate caspase-9 and remain resistant to apoptosis in response to Apo2L/TRAIL. Conversely, reduction of the Bcl-x_L/tBID ratio by CK2 inhibitors sensitizes such cancers to Apo2L/TRAIL-induced activation of caspase-9 via activation of BAX. We further demonstrate that the enhancement of Apo2L/TRAIL-induced tumor-cell death by CK2 inhibitors requires BAX, but not p53.

CK2 is active in rapidly proliferating tissues, and its activity is increased in response to diverse growth stimuli including insulin, insulin-like growth factor-1, epidermal growth factor, and androgens (9). A variety of human cancers and transformed cell lines exhibit constitutively high CK2 activity (9). Overexpression of the catalytic α subunit of CK2 in transgenic mice leads to T-cell lymphoma (10), and CK2α overexpression accelerates lymphomagenesis caused by loss of p53 (25). Although these observations have identified a role of CK2 in cell growth and neoplastic transformation, our findings suggest that CK2 also plays an instrumental role in protecting cancer cells from Apo2L/TRAIL-induced apoptosis. This mechanism of resistance may frequently operate in diverse tumor types with constitutive activation of CK2 via genetic aberrations, such as overexpression of receptor tyrosine kinases (HER2/neu, epidermal growth factor receptor, insulin-like growth factor-1 receptor). Our results indicate that such resistant cancers can be rendered sensitive to Apo2L/TRAIL-induced death by CK2 inhibitors. DRB, apigenin, and emodin competitively inhibit CK2 catalytic activity by directly interacting with the nucleotide-binding sites of subunits of CK2 (12–14). Apigenin is a plant flavonoid that is a major constituent of herbal chamomile and is found naturally in many fruits, vegetables, and plant-derived beverages (chamomile tea and wine). Emodin is a plant anthraquinone derivative isolated from *Rheum Palmatum*. Although our results provide a biological rationale for combining TRAIL/Apo2L with CK2 inhibitors such as apigenin or emodin for treatment of cancers, further studies are required to evaluate and optimize the therapeutic ratio of such regimens. In addition to defining CK2 as a key molecular determinant of the resistance of cancers to TRAIL/Apo2L-induced apoptosis, our findings may aid the development and genotype-specific application of TRAIL/Apo2L-based combinatorial regimens for the treatment of diverse human cancers.

Acknowledgments

We thank Dr. Bert Vogelstein for his kind gift of isogenic HCT116 cells and Dr. Céline Gelinas for kindly providing the *bcl-x*-CAT reporter plasmids.

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Appendix 5: Unpublished Data.

Figures 1-4

Ravi, R., Prouser, T and Bedi, A. Sensitization of breast cancer cells to death receptor-induced apoptosis by inhibition of NF-κB: Synergistic action of Apo2L/TRAIL, Interferon-γ, Aspirin and Apigenin.

Abstract presented at Era of Hope, Orlando, FL, September 2002 (Manuscript in preparation)

ABSTRACT

Although Apo2L/TRAIL is a promising anticancer agent, several breast cancer cell lines remain resistant to Apo2L/TRAIL even though they express death receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5. Our findings demonstrate that cancer cells can be sensitized to Apo2L/TRAIL-induced death by interferon-γ-mediated upregulation of BAK, caspase-8, and caspase-7. The elevation of procaspase-8 potentiates Apo2L/TRAIL-mediated formation of tBID, which then interacts with the more abundant BAK to implement mitochondrial outer membrane permeabilization (MOMP) and caspase-9 activation even in the absence of BAX. Interferon-γ also facilitates caspase-9-mediated apoptotic signaling downstream of MOMP by increasing the amount of procaspase-7. While interferon-γ potentiates death receptor-induced apoptosis, Apo2L/TRAIL death signaling is counteracted by expression of NF-κB-inducible survival proteins, such as Bcl-x_L and IAPs (cIAP-2 and XIAP). Many breast cancers exhibit constitutively high NF-κB activity resulting from phosphorylation of IκB by IκB kinase (IKK) and/or casein kinase II (CK2). Our findings demonstrate that simultaneous inhibition of IKKβ (with acetyl salicylic acid, ASA), and CK2 (with the plant flavonoid, apigenin), results in loss of NF-κB-dependent expression of Bcl-x_L and IAPs, thereby potentiating activation of caspases-9 and -7, and promoting tumor cell apoptosis in response to Apo2L/TRAIL. We also show that the reduction of NF-κB-induced survival proteins by ASA and apigenin synergizes with interferon-γ–mediated elevation of death signaling proteins to augment Apo2L/TRAIL-induced apoptosis of breast cancer cells. Further studies are required to evaluate and optimize the therapeutic ratio of the combinatorial regimen of Apo2L/TRAIL, interferon-γ, aspirin, and apigenin for treatment of breast cancers.

INTRODUCTION

Genetic aberrations that render cells incapable of executing apoptosis underlie the observed resistance of human breast cancers to anticancer agents. Unraveling mechanisms to unleash the apoptotic program in tumor cells could provide effective therapeutic interventions against breast cancers.

Tumor cell death can be triggered by engagement of specific death receptors belonging to the tumor necrosis factor receptor gene superfamily with the "death ligand", Apo2L/TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). Apo2L/TRAIL-induced cell death involves caspase-8-mediated cleavage of BID to form truncated BID (tBID). tBID induces activation of BAX or BAK leading to mitochondrial outer membrane permeabilization (MOMP) and release of a cocktail of pro-death cofactors (such as cytochrome c, Smac/DIABLO) into the cytoplasm. The interaction of cytochrome c with Apaf-1 results in a nucleotide-dependent conformational change that allows binding and transactivation of caspase-9, which in turn, activates downstream caspases such as caspase-3 and caspase-7. The activation of caspases 9, 3, and 7, is further potentiated by Smac/DIABLO (second mitochondria-derived activator of caspase), a protein which binds and antagonizes the IAPs.

Apo2L/TRAIL induces apoptosis of many cancer cell lines *in vitro*, and its tumoricidal activity and safety *in vivo* has been confirmed in preclinical animal models of human breast cancer xenografts. However, many breast cancer cell lines express death receptors for Apo2L/TRAIL, yet remain relatively resistant to Apo2L/TRAIL-induced apoptosis. The identification of the molecular determinants of Apo2L/TRAIL-induced death and key survival proteins that interrupt death receptor-induced signaling in tumor cells could aid the design of Apo2L/TRAIL-based combination regimens against breast cancers.

RESULTS

1. Tumor cell resistance to Apo2L/TRAIL-mediated apoptosis via loss of BAX, but not p53.

HCT116 cells have wild type *p53* ($p53^{+/+}$) and an intact *BAX* allele ($BAX^{+/-}$), and express functional p53 and BAX proteins. Isogenic p53-deficient ($p53^{-/-}$) or BAX-deficient ($BAX^{-/-}$) derivatives of HCT116 cells were generated by targeted inactivation of either both *p53* alleles or the wild-type *BAX* allele in a BAX heterozygote (8, 9). Exposure of both BAX-proficient ($p53^{-/-}$ and $p53^{+/+}$) and $BAX^{-/-}$ HCT116 cells to Apo2L/TRAIL resulted in activation of caspase-8 and caspase-8-mediated proteolysis of BID (Fig. 1, 2). The formation of truncated BID (tBID) by Apo2L/TRAIL triggered the mitochondrial activation of caspase-9, and resulted in cleavage of caspase-7 and PARP in BAX-proficient HCT116 cells ($p53^{+/+}$ or $p53^{-/-}$)(Fig. 1, Fig. 2). In contrast, isogenic $BAX^{-/-}$ HCT116 cells failed to activate caspase-9 or caspase-7, and were resistant to Apo2L/TRAIL-induced apoptosis (Fig. 1, 2, 4). Therefore, Apo2L/TRAIL-induced apoptosis of cancer cells is independent of *p53*, but requires BAX.

2. Interferon-γ augments the Apo2L/TRAIL-induced death signaling pathway.

We examined the effect of interferon-γ on expression of the molecular components of the Apo2L/TRAIL-induced death signaling pathway in $BAX^{+/-}$ or $BAX^{-/-}$ isogenic tumor cells. Immunoblot analyses demonstrated that treatment with interferon-γ increased expression of the zymogens, caspase-8 and caspase-7, in both $BAX^{+/-}$ and $BAX^{-/-}$cells, but did not change expression of caspase-9 (Fig. 2). Treatment with interferon-γ also increased expression of BAK, without altering levels of BAX (Fig. 2). Since interferon-γ augmented expression of sequential determinants of the Apo2L/TRAIL-induced death signaling pathway (caspase-8, BAK, and caspase-7), we investigated whether interferon-γ can overcome the resistance of $BAX^{-/-}$ tumor cells to Apo2L/TRAIL. Pre-incubation of either $BAX^{+/-}$ or $BAX^{-/-}$ tumor cells with interferon-γ for 16h (and continued exposure for 48h in the presence of Apo2L/TRAIL) promoted formation of tBID, activation of caspase-9 and caspase-7, efficient cleavage of PARP, and induction of tumor cell death in response to Apo2L/TRAIL (Fig. 2, 4).

3. Inhibition of Apo2L/TRAIL-induced apoptosis of tumor cells by Bcl-x_L

tBID triggers mitochondrial outer membrane permeabilization (MOMP) by inducing the allosteric activation of BAK or BAX. To investigate whether the induction of apoptosis by the combination of Apo2L/TRAIL and interferon-γ is hindered by Bcl-x_L, we introduced a retroviral vector encoding Bcl-x_L into BAX-proficient HCT116 cells [Bcl-x_L($BAX^{+/-}$)]. Although Apo2L/TRAIL (with or without interferon-γ) induced formation of tBID, it could not activate caspases-9 or -7, and failed to induce apoptosis in BAX-proficient tumor cells overexpressing exogenous Bcl-x_L [Bcl-x_L($BAX^{+/-}$)] (Fig. 2, 4). Therefore, the ability of tBID to activate BAX or BAK is curtailed via its sequestration by Bcl-x_L.

4. Apo2L/TRAIL-induced apoptosis is augmented by inhibiting NF-κB-dependent expression of Bcl-x_L and IAPs with acetyl salicylic acid and apigenin.

The human *bcl-x* promoter contains a κB DNA site (TTTACTGCCC; 298/+22) responsible for its Rel-dependent induction. In addition to Bcl-x_L, members of the inhibitor of apoptosis family [cIAP-2 and X-chromosome linked IAP (XIAP)] are also NF-κB-induced proteins which inhibit caspases (-9, -7, -3). Activation of NF-κB requires phosphorylation of the inhibitory proteins, the IκBs, by either the IκB kinase (IKK) complex or casein kinase II (CK2). The IKKβ catalytic subunit of IKK is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), such as acetyl salicylic acid (aspirin) or sulindac sulfide, whereas CK2 is inhibited by the plant flavone, apigenin (Fig. 3). The combination of ASA (1 mM) with apigenin (10 μM) resulted in a greater repression of NF-κB DNA-binding activity and endogenous Bcl-x_L and IAPs (cIAP-2 and XIAP) than either agent alone (Fig. 3). Consistent with the reduced levels of Bcl-x_L and IAPs, treatment with the combination of ASA with apigenin potentiated activation of caspases-9 and -7, and induction of tumor cell apoptosis in response to Apo2L/TRAIL (Fig. 2, 4).

5. Sensitization of breast cancer cells to Apo2L/TRAIL-induced apoptosis by the synergistic effects of IFN-γ and NF-κB inhibitors (aspirin and apigenin)

Our results indicate that interferon-γ enhances expression of members of the Apo2L/TRAIL-death signaling pathway (caspase-8, BAK, and caspase-7), while the expression of NF-κB-induced survival proteins (Bcl-x_L and IAPs) is reduced by the combination of ASA and apigenin (Fig 2, 3). To investigate whether interferon-γ–mediated elevation of death signaling proteins can synergize with the reduction of NF-κB-induced survival proteins to augment Apo2L/TRAIL-induced apoptosis, we examined the effect of a combinatorial regimen of interferon-γ, ASA, and apigenin, on the sensitivity of human breast cancer cell lines (MCF-7, SKBr-3, Hs578) to Apo2L/TRAIL-induced death. All three cell lines were sensitized to Apo2L/TRAIL-induced apoptosis by the synergistic effects of interferon-γ and NF-κB inhibitors (ASA, and apigenin)(Fig. 4).

DISCUSSION

Human breast cancer cell lines exhibit a wide heterogeneity in their sensitivity to TRAIL/Apo2L *in vitro*, and many remain resistant to Apo2L/TRAIL-induced apoptosis. These data suggest that successful treatment of breast cancers with TRAIL/Apo2L may require its combination with agents that inhibit survival signals responsible for protecting tumor cells from death receptor-induced apoptosis.

Apo2L/TRAIL-induced cell death involves caspase-8-mediated cleavage of BID to form truncated BID (tBID). tBID induces activation of BAX or BAK leading to mitochondrial outer membrane permeabilization (MOMP) and release of a cocktail of pro-death cofactors (such as cytochrome c, Smac/DIABLO) into the cytoplasm. Since many breast cancer cells exhibit decreased expression of BAX (*unpublished observations*), our data suggest that BAX-deficiency may render breast cancer cells resistant to Apo2L/TRAIL-induced apoptosis. In addition, amplification and consequent overexpression c-erbB2 (HER-2/neu) or IGF-1 receptor (IGF-1R) is observed in a significant proportion of human breast cancers. Both HER-2/neu and IGF-1R promote PI3 kinase (PI3-K)-mediated phosphorylation and activation of Akt, a serine-threonine kinase that, in turn, activates the IκB kinase (IKK) complex. The activated IKK complex induces phosphorylation-mediated degradation of IκB, thereby promoting activation of NF-κB. In addition to aberrant activation of the IKK complex, breast cancers frequently exhibit increased activity of casein kinase II (CK2). The activation of either IKK or CK2 results in constitutive NF-κB activity in breast cancer cells. Our results indicate that NF-κB protects breast cancer cells from Apo2L/TRAIL-induced apoptosis by promoting expression of Bcl-x_L, a Bcl-2 family member that sequesters tBID and inhibits activation of BAX. In addition to Bcl-x_L, NF-kB also protects tumor cells from Apo2L/TRAIL-induced apoptosis by inducing expression of members of the inhibitor of apoptosis family [cIAP-2 and X-chromosome linked IAP (XIAP)].

Our findings demonstrate that cancer cells can be sensitized to Apo2L/TRAIL-induced death by interferon-γ-mediated upregulation of BAK, caspase-8, and caspase-7. The elevation of procaspase-8 potentiates Apo2L/TRAIL-mediated formation of tBID, which then interacts with the more abundant BAK to implement mitochondrial outer membrane permeabilization (MOMP) and caspase-9 activation even in the absence of BAX. Interferon-γ also facilitates caspase-9-mediated apoptotic signaling downstream of MOMP by increasing the amount of procaspase-7. While interferon-γ potentiates death receptor-induced apoptosis, Apo2L/TRAIL death signaling is counteracted by expression of NF-κB-inducible survival proteins, such as Bcl-x_L and IAPs (cIAP-2 and XIAP). Our findings also demonstrate that simultaneous inhibition of IKKβ (with acetyl salicylic acid, ASA), and CK2 (with the plant flavonoid, apigenin), results in loss of NF-κB-dependent expression of Bcl-x_L and IAPs, thereby potentiating activation of caspases-9 and -7, and promoting tumor cell apoptosis in response to Apo2L/TRAIL. We also show that the reduction of NF-κB-induced survival proteins by ASA and apigenin synergizes with interferon-γ–mediated elevation of death signaling proteins to augment Apo2L/TRAIL-induced apoptosis of breast cancer cells (Fig. 4).

Our results suggest that the following Apo2L/TRAIL-based combination regimens may be useful for the treatment of human breast cancers:

1. Apo2L/TRAIL + Interferon-γ + inhibitors of growth factor receptor tyrosine kinases (HER-2/neu) (trastuzumab).
2. Apo2L/TRAIL + interferon-γ + aspirin + apigenin
3. Apo2L/TRAIL + interferon-g + NF-κB inhibitors (PS-341 or parthenolide)

Further studies are required to evaluate and optimize the therapeutic ratio of these combinatorial regimens.

Sensitization of breast cancers to death receptor-induced apoptosis by inhibition of NF-κB: Synergistic action of Apo2L/TRAIL, Interferon-γ, Aspirin, and Apigenin

Rajani Ravi, Ph.D., Traci S. Prouser, and Atul Bedi, M.D. The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland, U.S.A.

ABSTRACT

Although Apo2L/TRAIL is a promising anticancer agent, several breast cancer cell lines remain resistant to Apo2L/TRAIL even though they express death receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5. Our findings demonstrate that cancer cells can be sensitized to Apo2L/TRAIL-induced death by interferon-γ-mediated upregulation of BAK, caspase-8, and caspase-7. The elevation of procaspase-8 potentiates Apo2L/TRAIL-mediated formation of tBID, which then interacts with the more abundant BAK to implement mitochondrial outer membrane permeabilization (MOMP) and caspase-9 activation even in the absence of BAX. Interferon-γ also facilitates caspase-9-mediated apoptotic signaling downstream of MOMP by increasing the amount of procaspase-7. While interferon-γ potentiates death receptor-induced apoptosis, Apo2L/TRAIL death signaling is counteracted by expression of NF-κB-inducible survival proteins, such as Bcl-x_L and IAPs (cIAP-2 and XIAP). Many breast cancers exhibit constitutively high NF-κB activity resulting from phosphorylation of IκB by IκB kinase (IKK) and/or casein kinase II (CK2). Our findings demonstrate that simultaneous inhibition of IKKβ (with acetyl salicylic acid, ASA), and CK2 (with the plant flavonoid, apigenin), results in loss of NF-κB-dependent expression of Bcl-x_L and IAPs, thereby potentiating activation of caspases-9 and -7, and promoting tumor cell apoptosis in response to Apo2L/TRAIL. We also show that the reduction of NF-κB-induced survival proteins by ASA and apigenin synergizes with interferon-γ–mediated elevation of death signaling proteins to augment Apo2L/TRAIL-induced apoptosis of breast cancer cells. Further studies are required to evaluate and optimize the therapeutic ratio of the combinatorial regimen of Apo2L/TRAIL, interferon-γ, aspirin, and apigenin for treatment of breast cancers.

INTRODUCTION

Genetic aberrations that render cells incapable of executing apoptosis underlie the observed resistance of human breast cancers to anticancer agents. Unraveling mechanisms to unleash the apoptotic program in tumor cells could provide effective therapeutic interventions against breast cancers.

Tumor cell death can be triggered by engagement of specific death receptors belonging to the tumor necrosis factor receptor gene superfamily with the "death ligand", Apo2L/TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). Apo2L/TRAIL-induced cell death involves caspase-8-mediated cleavage of BID to form truncated BID (tBID). tBID induces activation of BAX or BAK leading to mitochondrial outer membrane permeabilization (MOMP) and release of a cocktail of pro-death cofactors (such as cytochrome c, Smac/DIABLO) into the cytoplasm. The interaction of cytochrome c with Apaf-1 results in a nucleotide-dependent conformational change that allows binding and transactivation of caspase-9, which in turn, activates downstream caspases such as caspase-3 and caspase-7. The activation of caspases 9, 3, and 7, is further potentiated by Smac/DIABLO (second mitochondria-derived activator of caspase), a protein which binds and antagonizes the IAPs.

Apo2L/TRAIL induces apoptosis of many cancer cell lines *in vitro*, and its tumoricidal activity and safety *in vivo* has been confirmed in preclinical animal models of human breast cancer xenografts. However, many breast cancer cell lines express death receptors for Apo2L/TRAIL, yet remain relatively resistant to Apo2L/TRAIL-induced apoptosis. The identification of the molecular determinants of Apo2L/TRAIL-induced death and key survival proteins that interrupt death receptor-induced signaling in tumor cells could aid the design of Apo2L/TRAIL-based combination regimens against breast cancers.

RESULTS

1. Tumor cell resistance to Apo2L/TRAIL-mediated apoptosis via loss of BAX, but not p53.

HCT116 cells have wild type *p53* ($p53^{+/+}$) and an intact *BAX* allele ($BAX^{+/+}$), and express functional p53 and BAX proteins. Isogenic p53-deficient ($p53^{-/-}$) or BAX-deficient ($BAX^{-/-}$) derivatives of HCT116 cells were generated by targeted inactivation of either both *p53* alleles or the wild-type *BAX* allele in a BAX heterozygote (8, 9). Exposure of both BAX-proficient ($p53^{+/+}$ and $p53^{-/-}$) and $BAX^{-/-}$ HCT116 cells to Apo2L/TRAIL resulted in activation of caspase-8 and caspase-8-mediated proteolysis of BID (Fig. 1, 2). The formation of truncated BID (tBID) by Apo2L/TRAIL triggered the mitochondrial activation of caspase-9, and resulted in cleavage of caspase-7 and PARP in BAX-proficient HCT116 cells ($p53^{+/+}$ or $p53^{-/-}$)(Fig. 1, Fig. 2). In contrast, isogenic $BAX^{-/-}$ HCT116 cells failed to activate caspase-9 or caspase-7, and were resistant to Apo2L/TRAIL-induced apoptosis (Fig. 1, 2, 4). Therefore, Apo2L/TRAIL-induced apoptosis of cancer cells is independent of *p53*, but requires BAX.

2. Interferon-γ augments the Apo2L/TRAIL-induced death signaling pathway.

We examined the effect of interferon-γ on expression of the molecular components of the Apo2L/TRAIL-induced death signaling pathway in $BAX^{+/-}$ or $BAX^{-/-}$ isogenic tumor cells. Immunoblot analyses demonstrated that treatment with interferon-γ increased expression of the zymogens, caspase-8 and caspase-7, in both $BAX^{+/-}$ and $BAX^{-/-}$cells, but did not change expression of caspase-9 (Fig. 2). Treatment with interferon-γ also increased expression of BAK, without altering levels of BAX (Fig. 2). Since interferon-γ augmented expression of sequential determinants of the Apo2L/TRAIL-induced death signaling pathway (caspase-8, BAK, and caspase-7), we investigated whether interferon-γ can overcome the resistance of $BAX^{-/-}$ tumor cells to Apo2L/TRAIL. Pre-incubation of either $BAX^{+/-}$ or $BAX^{-/-}$ tumor cells with interferon-γ for 16h (and continued exposure for 48h in the presence of Apo2L/TRAIL) promoted formation of tBID, activation of caspase-9 and caspase-7, efficient cleavage of PARP, and induction of tumor cell death in response to Apo2L/TRAIL (Fig. 2, 4).

3. Inhibition of Apo2L/TRAIL-induced apoptosis of tumor cells by Bcl-x_L

tBID triggers mitochondrial outer membrane permeabilization (MOMP) by inducing the allosteric activation of BAK or BAX. To investigate whether the induction of apoptosis by the combination of Apo2L/TRAIL and interferon-γ is hindered by Bcl-x_L, we introduced a retroviral vector encoding Bcl-x_L into BAX-proficient HCT116 cells [Bcl-x_L($BAX^{+/-}$)]. Although Apo2L/TRAIL (with or without interferon-γ) induced formation of tBID, it could not activate caspases-9 or -7, and failed to induce apoptosis in BAX-proficient tumor cells overexpressing exogenous Bcl-x_L [Bcl-x_L($BAX^{+/-}$)] (Fig. 2, 4). Therefore, the ability of tBID to activate BAX or BAK is curtailed via its sequestration by Bcl-x_L.

4. Apo2L/TRAIL-induced apoptosis is augmented by inhibiting NF-κB-dependent expression of Bcl-x_L and IAPs with acetyl salicylic acid and apigenin.

The human *bcl-x* promoter contains a κB DNA site (TTTACTGCCC; 298/+22) responsible for its Rel-dependent induction. In addition to Bcl-x_L, members of the inhibitor of apoptosis family [cIAP-2 and X-chromosome linked IAP (XIAP)] are also NF-κB-induced proteins which inhibit caspases (-9, -7, -3). Activation of NF-κB requires phosphorylation of the inhibitory proteins, the IκBs, by either the IκB kinase (IKK) complex or casein kinase II (CK2). The IKKβ catalytic subunit of IKK is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), such as acetyl salicylic acid (aspirin) or sulindac sulfide, whereas CK2 is inhibited by the plant flavone, apigenin (Fig. 3). The combination of ASA (1 mM) with apigenin (10 μM) resulted in a greater repression of NF-κB DNA-binding activity and endogenous Bcl-x_L and IAPs (cIAP-2 and XIAP) than either agent alone (Fig. 3). Consistent with the reduced levels of Bcl-x_L and IAPs, treatment with the combination of ASA with apigenin potentiated activation of caspases-9 and -7, and induction of tumor cell apoptosis in response to Apo2L/TRAIL (Fig. 2, 4).

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Our results indicate that interferon-γ enhances expression of members of the Apo2L/TRAIL-death signaling pathway (caspase-8, BAK, and caspase-7), while the expression of NF-κB-induced survival proteins (Bcl-x_L and IAPs) is reduced by the combination of ASA and apigenin (Fig 2, 3). To investigate whether interferon-γ–mediated elevation of death signaling proteins can synergize with the reduction of NF-κB-induced survival proteins to augment Apo2L/TRAIL-induced apoptosis, we examined the effect of a combinatorial regimen of interferon-γ, ASA, and apigenin, on the sensitivity of human breast cancer cell lines (MCF-7, SKBr-3, Hs578) to Apo2L/TRAIL-induced death. All three cell lines were sensitized to Apo2L/TRAIL-induced apoptosis by the synergistic effects of interferon-γ and NF-κB inhibitors (ASA, and apigenin)(Fig. 4).

CONCLUSIONS

The reduction of NF-κB-induced survival proteins by aspirin and apigenin synergizes with interferon-γ–mediated elevation of death signaling proteins to augment Apo2L/TRAIL-induced apoptosis of breast cancer cells (Fig. 4). The combination of Apo2L/TRAIL, interferon-γ, aspirin, and apigenin may be an effective regimen for treatment of breast cancers.

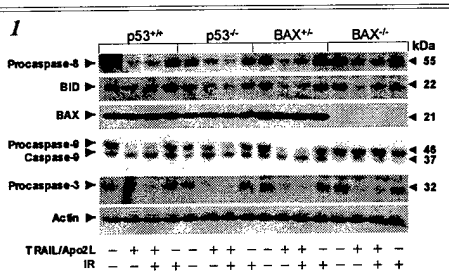


Fig 1. Tumor cell resistance to Apo2L/TRAIL-induced apoptosis via loss of BAX, but not p53. Western-blot analyses of whole cell lysates after treatment of cells with Apo2L/TRAIL (100 ng/ml), ionizing radiation (IRX5 Gy), or the combination of Apo2L/TRAIL and IR, as indicated.

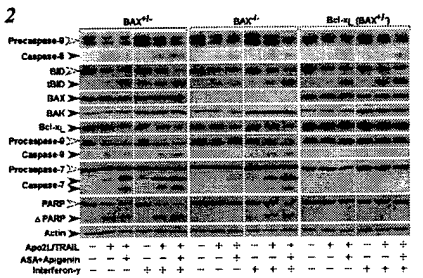


Fig 2. Interferon-γ augments the Apo2L/TRAIL-induced death signaling; Synergy with inhibition of NF-κB-dependent expression of Bcl-x_L by acetyl salicylic acid and apigenin. Western-blot analyses after 12h of treatment with Apo2L/TRAIL (100 ng/ml), acetyl salicylic acid (ASA)(1 mM), apigenin (10 μM), and/or interferon-γ (1000 U/ml; pre-treatment for 16h). Open arrow heads - inactive procaspases; filled arrow heads - processed active forms of caspases.

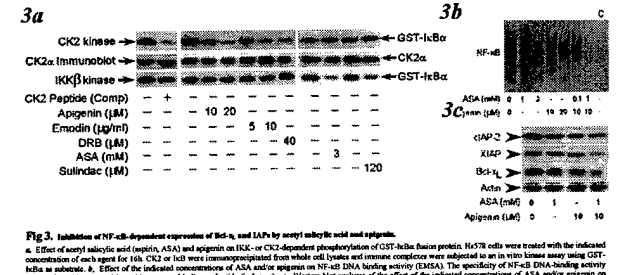
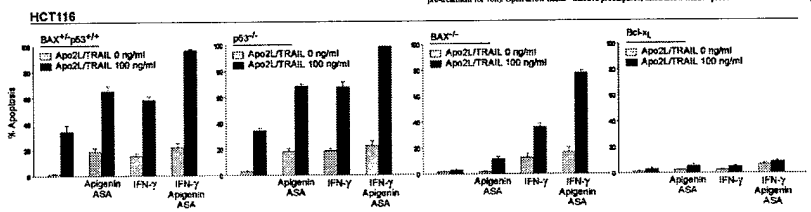
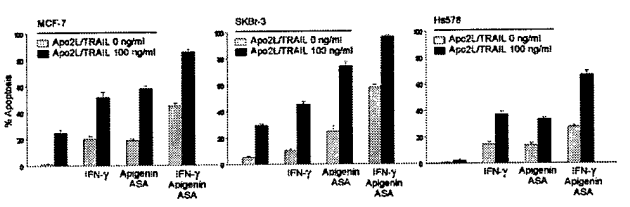
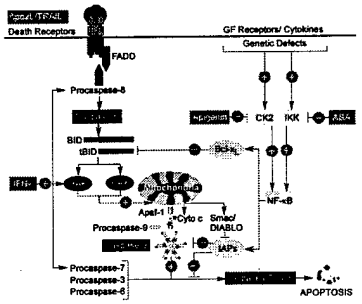


Fig 3. Inhibition of NF-κB-dependent expression of Bcl-x_L and IAPs by acetyl salicylic acid and apigenin. a, Effect of acetyl salicylic acid (aspirin, ASA) and apigenin on IKK- or CK2-dependent phosphorylation of GST-IκBα fusion protein. Hs578 cells were treated with the indicated concentration of each agent for 16h. CK2 or IκBα were immunoprecipitated from whole cell lysates and immune complexes were subjected to an in vitro kinase assay using GST-IκBα as substrate. *b*, Effect of the indicated concentrations of ASA and/or apigenin on NF-κB DNA binding activity (EMSA). The specificity of NF-κB DNA-binding activity was confirmed by competition with excess cold oligonucleotide (in lane c). *c*, Western-blot analyses of the effect of the indicated concentrations of ASA and/or apigenin on expression of Bcl-x_L, cIAP-2 and XIAP.







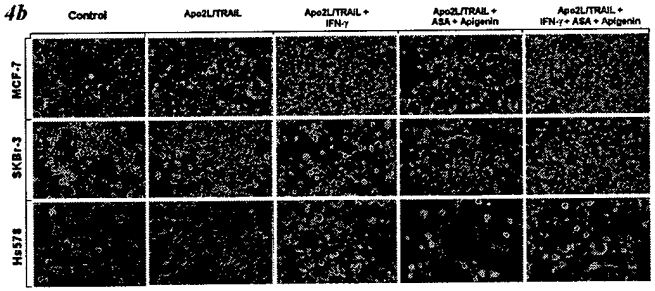


Fig 4. Interferon-γ synergizes with acetyl salicylic acid and apigenin to augment Apo2L/TRAIL-induced tumor cell apoptosis. a, Quantification of the percentage of cell death in HCT116 cells and breast cancer cell lines (MCF-7, SKBr-3, Hs578) following 48h of treatment with Apo2L/TRAIL (100 ng/ml), ASA (1 mM), apigenin (10 μM), and/or interferon-γ (1000 U/ml; pre-treatment for 16h), as indicated (mean of three independent experiments; *bars*, ± SE). *b*, Phase contrast photomicrographs of breast cancer cells after 48h of treatment with Apo2L/TRAIL (100 ng/ml), ASA (1 mM), apigenin (10 μM), and/or interferon-γ (1000 U/ml; pre-treatment for 16h)], as indicated. *c*, Schematic representation of the sensitization of BAX-proficient as well as BAX-deficient cancers to Apo2L/TRAIL-induced apoptosis by interferon-γ, acetyl salicylic acid, and apigenin.

RESEARCH FUNDED BY:

U.S. Army Medical Research and Materiel Command – Department of Defense, Breast Cancer Research Program: Award Number DAMD17-99-1-9230

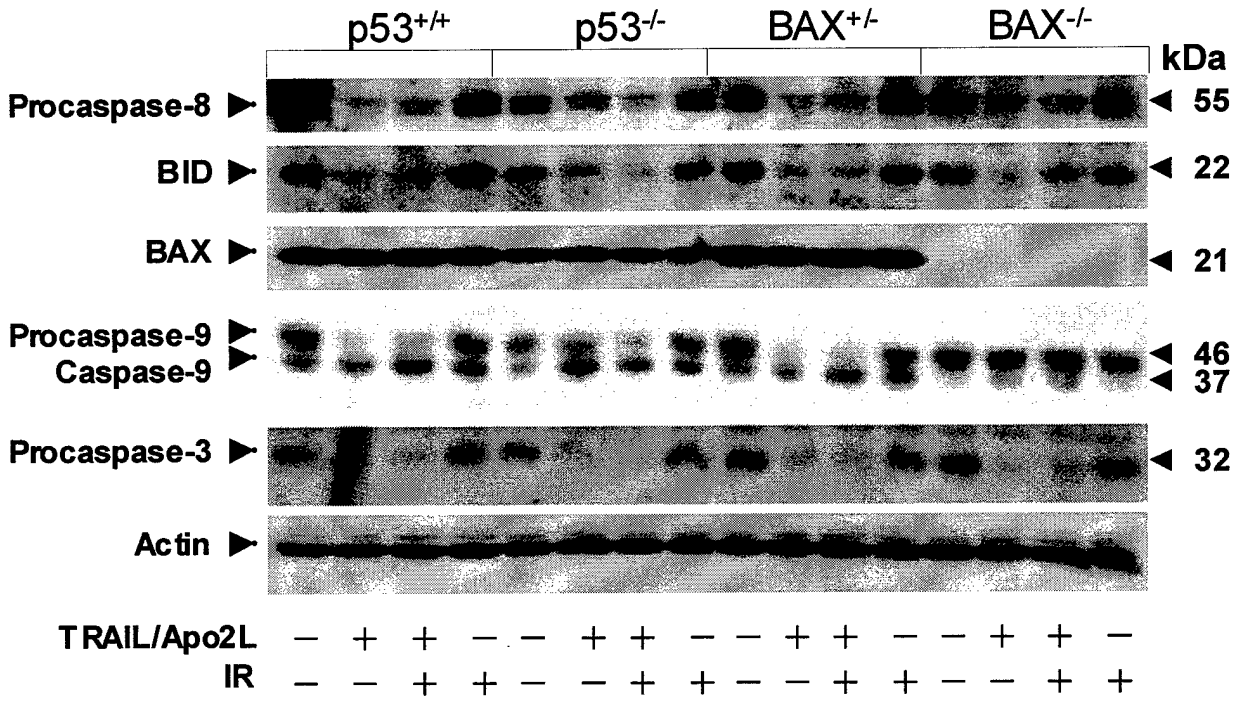


Fig 1. **Tumor cell resistance to Apo2L/TRAIL-induced apoptosis via loss of BAX, but not p53.**

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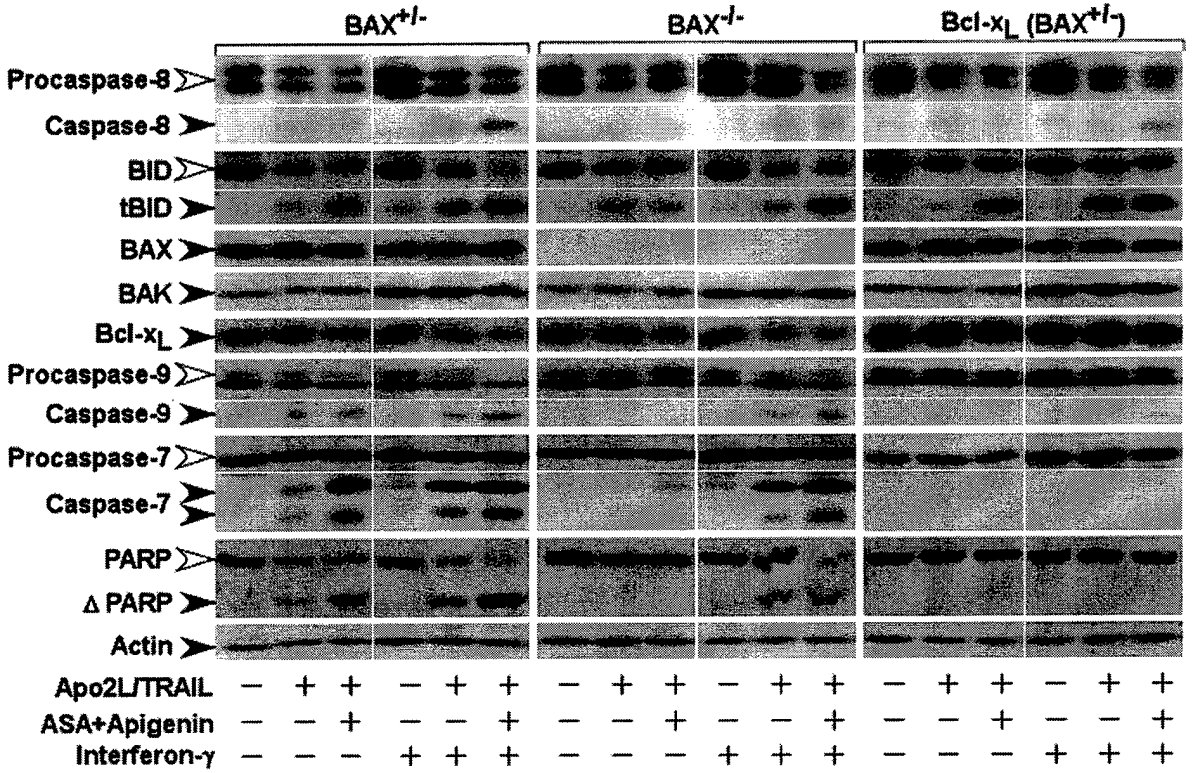

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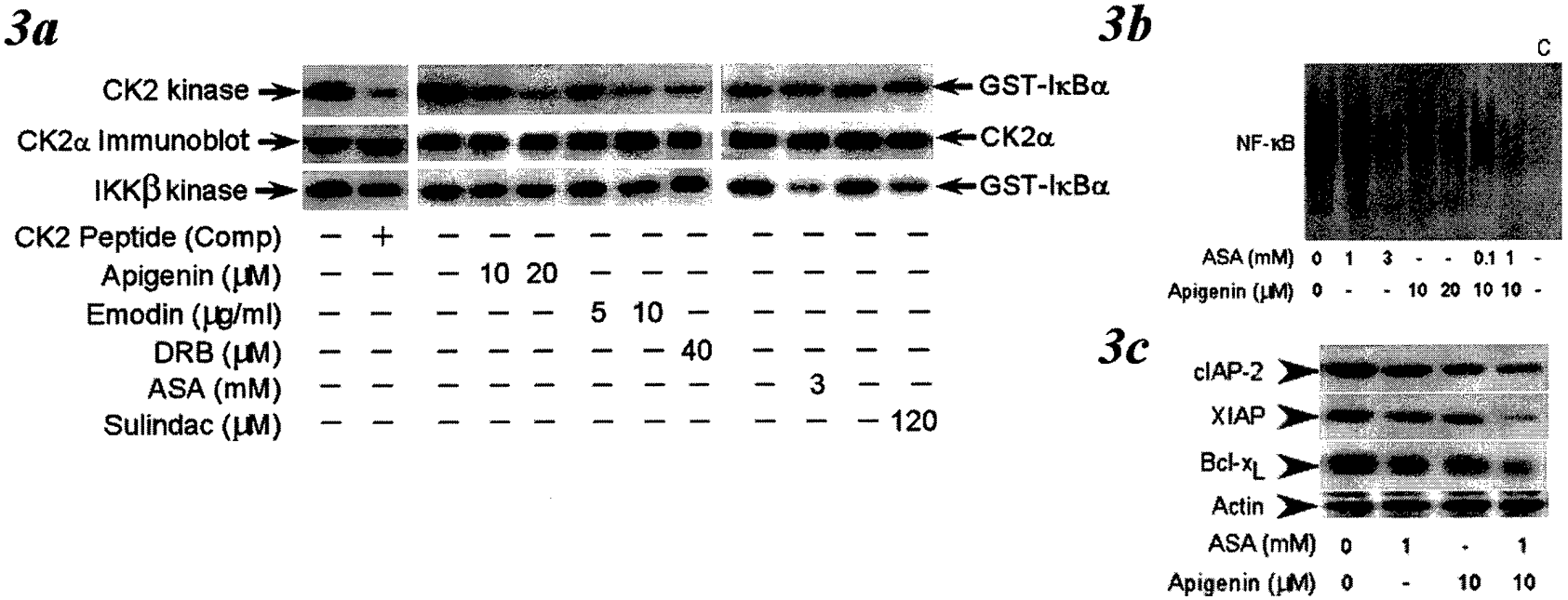

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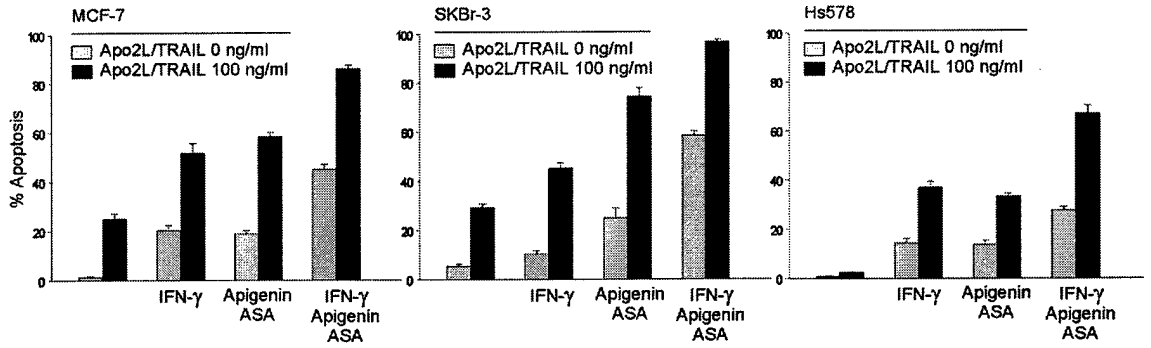
4a



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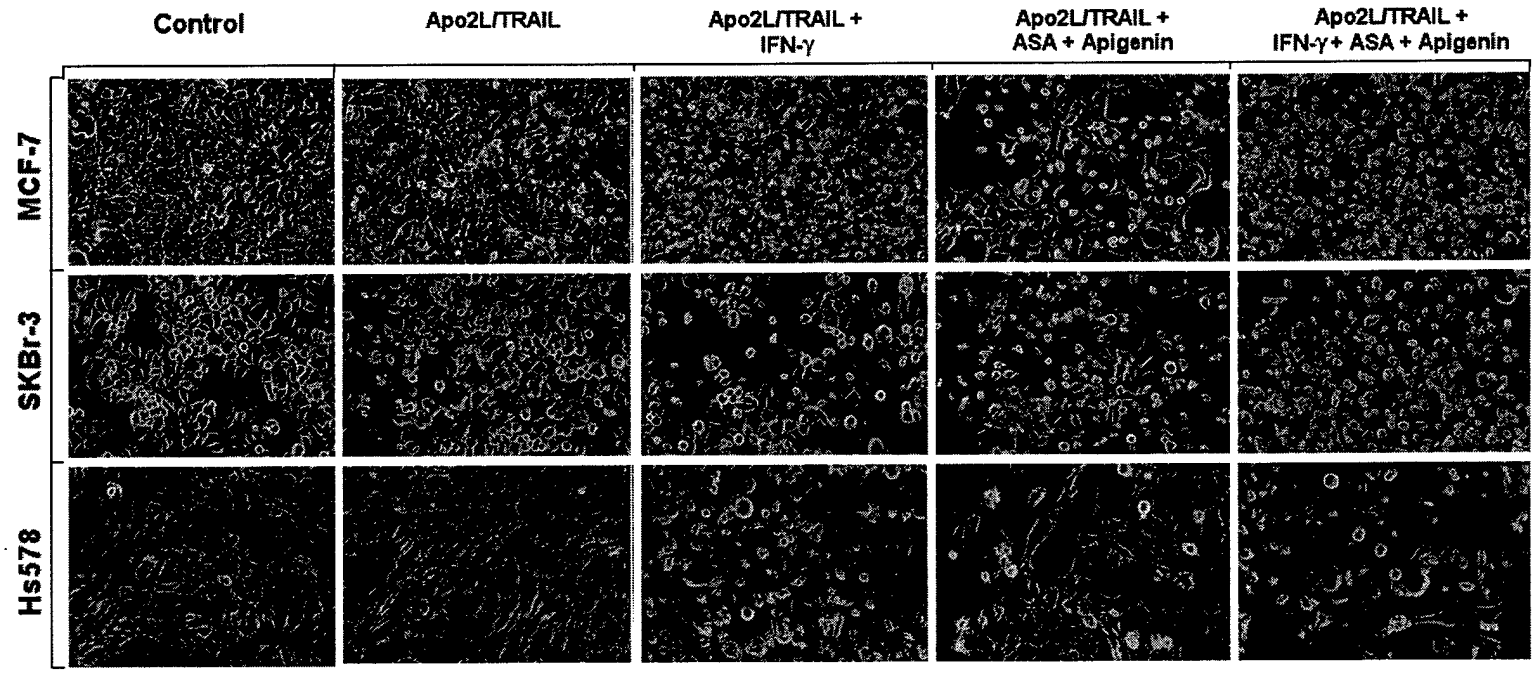

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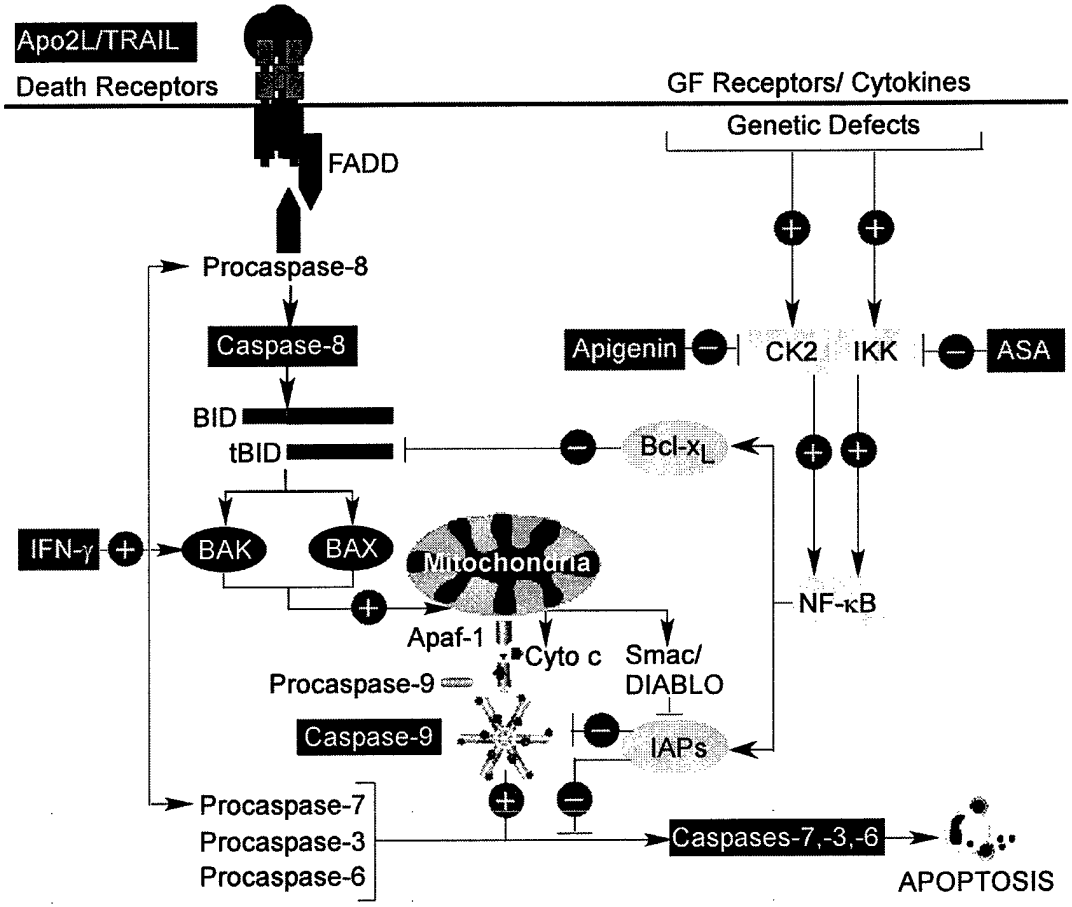
Apo2L/TRAIL
Death Receptors
GF Receptors/ Cytokines
Genetic Defects
FADD
Procaspase-8
Caspase-8
Apigenin
CK2
IKK
ASA
BID
tBID
Bcl-xL
IFN-γ
BAK
BAX
Mitochondria
NF-κB
Apaf-1
Cyto c
Smac/
DIABLO
Procaspase-9
Caspase-9
IAPs
Procaspase-7
Procaspase-3
Procaspase-6
Caspases-7,-3,-6
APOPTOSIS

Appendix 6: Reprint of publication:

Ravi, R and Bedi, A. Role of Death Receptors in Apoptosis, *Genetics of Apoptosis*. BIOS Scientific Publishers, Oxford, U.K.. Editor – Grimm, S., 2002 (In Press)

1. Death receptors in apoptosis

Rajani Ravi and Atul Bedi

1. Introduction

The requirement of cell death to preserve life is no paradox for multicellular animals. Programmed cell death, or apoptosis, enables the physiologic culling of excess cells during embryonic development and tissue remodeling or regeneration in adult animals. In addition to maintaining homeostasis by controlling cell number in proliferating tissues, apoptosis plays an instrumental role in the selective attrition of neurons that fail to establish functional synaptic connections during the development of vertebrate nervous systems. The vertebrate immune system uses apoptosis to delete lymphocytes with inoperative or autoreactive receptors from its repertoire, and to reverse clonal expansion at the end of an immune response. Cytotoxic T cells and natural killer cells induce apoptosis of target cells to effect innate and adaptive immune responses to intracellular pathogens, cancer cells, or transplanted tissues. The altruistic demise of cells in response to cellular stress or injury, or genetic errors, serves to preserve genomic integrity and constitutes an important mechanism of tumor surveillance.

Given the crucial role of apoptosis in such a diverse array of physiologic functions, it is no surprise that aberrations of this process underlie a host of developmental, immune, degenerative, and neoplastic disorders. This appreciation has fueled a furious investigation of the molecular determinants and mechanisms of apoptosis and a search for the key regulators of this process (Hengartner, 2000). The molecular execution of cell death involves activation of members of a family of cysteine-dependent aspartate-specific proteases (caspases) by two major mechanisms. One mechanism, termed the 'intrinsic' pathway, signals the release of prodeath factors from the mitochondria via the action of pro-apoptotic members of the Bcl-2 family (Green and Reed, 1998; Gross *et al.*, 1999a; Green, 2000). An alternative mechanism of activating caspases and mitochondrial disruption, termed the 'extrinsic' pathway, is triggered by engagement of cell-surface 'death receptors' by their specific ligands (Ashkenazi and Dixit, 1998).

In this chapter, we review our current understanding of the molecular determinants and mechanisms of death receptor-induced apoptosis, and identify the key regulators of these death-signaling pathways. We also review the physiologic role of death receptors and describe pathologic conditions that result from a failure or deregulation of their activity. Finally, we highlight the enormous promise of targeting death receptors or their regulatory circuits for treatment of human disease.

2. Death receptors and ligands

Death receptors are cell-surface receptors that trigger death signals following engagement with their cognate 'death ligands' (Ashkenazi and Dixit, 1998). Death receptor-transduced signals play an instrumental role in 'instructive apoptosis', a mechanism that has evolved to enable the deletion of cells in higher metazoans. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily, whose members have cysteine-rich extracellular domains (CRDs) in their amino terminal region (Smith *et al.*, 1994). The death receptors constitute a subgroup of this family that also possess a homologous cytoplasmic sequence termed the 'death domain' (DD) (Figure 1) (Brakebusch *et al.*, 1992; Itoh and Nagata, 1993). The best-characterized death receptors are TNFR1 (also termed p55 or CD120a) (Smith *et al.*, 1994), CD95 (also called Fas or Apo1) (Nagata, 1997), avian CAR1, death receptor 3 (DR3; also called Apo3, WSL-1, TRAMP, or LARD) (Chinnaiyan *et al.*, 1996b; Kitson *et al.*, 1996; Marsters *et al.*, 1996; Bodmer *et al.*, 1997; Screaton *et al.*, 1997b), TRAIL-R1 (also called DR4) (Pan *et al.*, 1997b), TRAIL-R2 (also called DR5, Apo2, TRICK2, or KILLER) (Pan *et al.*, 1997a; Screaton *et al.*, 1997a; Sheridan *et al.*, 1997; Walczak *et al.*, 1997; Wu, G.S. *et al.*, 1997), and DR6 (Pan *et al.*, 1998a). These receptors are activated by ligands of the TNF gene superfamily; TNFR1 is ligated by TNF and lymphotoxin α, CD95 is bound by CD95L (FasL) (Nagata, 1997), DR3 interacts with Apo3 ligand (Apo3L, also called TWEAK) (Chicheportiche *et al.*, 1997; Marsters *et al.*, 1998), and TRAIL-R1 and TRAIL-R2 are engaged by Apo2 ligand (Apo2L, which is also called TNF-related apoptosis-inducing ligand [TRAIL]) (Wiley *et al.*, 1995; Pitti *et al.*, 1996).

3. Induction of apoptosis by death receptors

3.1 The molecular machinery of cell death – caspases

The molecular machinery of cell death comprises an evolutionarily conserved family of cysteine aspartate proteases (caspases) that execute cell disassembly via cleavage of critical substrates that maintain cytoskeletal and DNA integrity (Earnshaw *et al.*, 1999). Caspases recognize specific tetrapeptide motifs in their target proteins and cleave their substrates at Asp-Xxx bonds (after aspartic acid residues) (Thornberry *et al.*, 1997). At least 14 caspases and more than 100 substrates have been identified (Earnshaw *et al.*, 1999). Caspases are divided into distinct subfamilies according to their structural and sequence identities, and their substrate specificity is determined by the specific pattern of four residues amino-terminal to the cleavage site (the P2–P4 positions). While caspases typically function by inactivat-

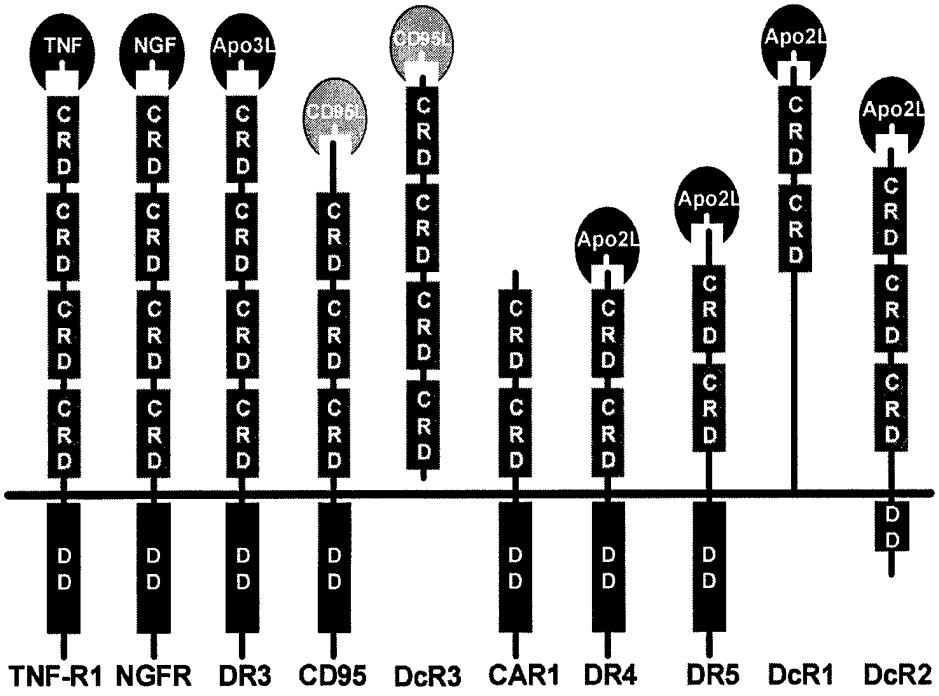

Figure 1. Death and decoy receptors of the TNF receptor family.

Death receptors have variable numbers of cysteine-rich extracellular domains (CRDs) in their extracellular ligand-binding amino terminal regions, and a homologous cytoplasmic sequence, termed the 'death domain' (DD), which is essential for apoptosis signaling. Death receptors are activated by ligands of the TNF gene superfamily; TNFR1 is ligated by TNFα, CD95/Fas is bound by CD95L, DR3 interacts with Apo3L, and TRAIL-R1 and TRAIL-R2 are engaged by Apo2L/TRAIL. DcR1 (TRAIL-R3) is structurally related to DR4 and DR5, but lacks a cytoplasmic tail. DcR2 (TRAIL-R4) also resembles DR4 and DR5, but has a truncated cytoplasmic DD. The extracellular domains of DcR1 and DcR2 compete with DR4 and DR5 for binding to Apo2L/TRAIL, but cannot initiate death signals in response to ligand-engagement. DcR3 binds to CD95L and inhibits CD95-CD95L interactions.

ing proteins by proteolytic cleavage, they can also, in some cases, activate the target by cleaving off a negative regulatory domain. Caspase-mediated cleavage of key substrates underlies many of the characteristic features of apoptosis such as nuclear shrinkage (Rao *et al.*, 1996; Buendia *et al.*, 1999), plasma membrane blebbing (Rudel and Bokoch, 1997; Coleman *et al.*, 2001; Sebbagh *et al.*, 2001), and internucleosomal DNA fragmentation (Liu, X. *et al.*, 1997; Enari *et al.*, 1998; Sakahira *et al.*, 1998). Deficiency of specific caspases or their inhibition can prevent the induction of apoptosis in response to diverse death stimuli (Earnshaw *et al.*, 1999). As such, caspases represent the fundamental executioners of cell death.

3.2 Activation of caspases by death receptors

Caspases are synthesized as inactive zymogens that comprise an N-terminal prodomain and two other domains, p20 and p10, which form the active mature enzyme upon cleavage between the p20 and p10 domains as well as between the

p20 domain and the prodomain (Earnshaw *et al.*, 1999). Since these Asp-X cleavage sites correspond to caspase substrate motifs, procaspases can be activated by either previously activated upstream caspases or by autocatalytic processing (Thornberry *et al.*, 1997).

Members of the death receptor family share the same fundamental mechanism(s) of activating caspases and amplifying this enzymatic cascade (*Figure 2*). These sequential steps and signaling pathways are dissected below:

Formation of the death-inducing signaling complex – activation of the initiator caspase-8/FLICE.

Death receptors are type I transmembrane proteins containing cytoplasmic sequences (DDs) that are essential for transduction of the apoptotic signal (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). The oligomerization of death receptors by engagement of their cognate ligands results in the rapid assembly of a membrane-bound death-inducing signaling complex (DISC) (Kischkel *et al.*, 1995). Ligand-induced trimerization of the CD95/Fas/Apo1 receptor facilitates binding of the adapter protein, FADD (Fas-associated DD protein; also known as mediator of receptor-induced toxicity [MORT1]) (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995) through homotypic interactions between their DD (Brakebusch *et al.*, 1992; Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). Receptor-bound FADD molecules form higher-order oligomers, or 'fibers' (Siegel *et al.*, 1998). FADD also carries a so-called death-effector domain (DED), which, in turn, interacts with the analogous DED motifs found in the N-terminal region of the zymogen form of caspase-8 (procaspase-8; also called FLICE or MACH) (Boldin *et al.*, 1996; Muzio *et al.*, 1996; Medema *et al.*, 1997). FADD-dependent recruitment and aggregation of multiple procaspase-8 molecules to the receptor/FADD scaffold results in autocatalytic cleavage and cross-activation by induced proximity, thereby releasing active caspase-8 into the cytoplasm (Martin *et al.*, 1998; Muzio *et al.*, 1998; Yang *et al.*, 1998a).

Other death receptors activate caspase-8 in a fashion analogous to that of CD95. While death receptors for Apo2L/TRAIL (TRAIL-R1/DR4 and TRAIL-R2/DR5) directly bind FADD, TNF-α-bound TNFR1 binds the adapter molecule TRADD (TNFR1-associated DD protein), which, in turn, recruits FADD to the receptor complex (Hsu *et al.*, 1996). Experiments with FADD gene-knockout mice (Zhang, L. *et al.*, 1998) or transgenic mice expressing a dominant-negative mutant of FADD (FADD-DN) in T cells (Newton *et al.*, 1998; Zornig *et al.*, 1998) have demonstrated that FADD is essential for induction of apoptosis by CD95/Fas, TNFR1, and DR3. However, a similar obligatory role of FADD in apoptosis signaling by Apo2L/TRAIL or its death receptors has not been uniformly observed. Cells from FADD-deficient mice remain susceptible to DR4-induced apoptosis (Yeh *et al.*, 1998), and ectopic expression of FADD-DN failed to block induction of apoptosis by either Apo2L/TRAIL or overexpression of either DR4 or DR5 (Pan *et al.*, 1997a; Sheridan *et al.*, 1997). While these studies suggest the existence of a FADD-independent mechanism by which Apo2L/TRAIL activates caspase-8 (perhaps through another adapter), other conflicting observations indicate that DR4- or DR5-induced apoptosis can be inhibited by transfection of FADD-DN (Walczak *et al.*, 1997). The physiologic role of FADD is further supported by Apo2L/TRAIL-

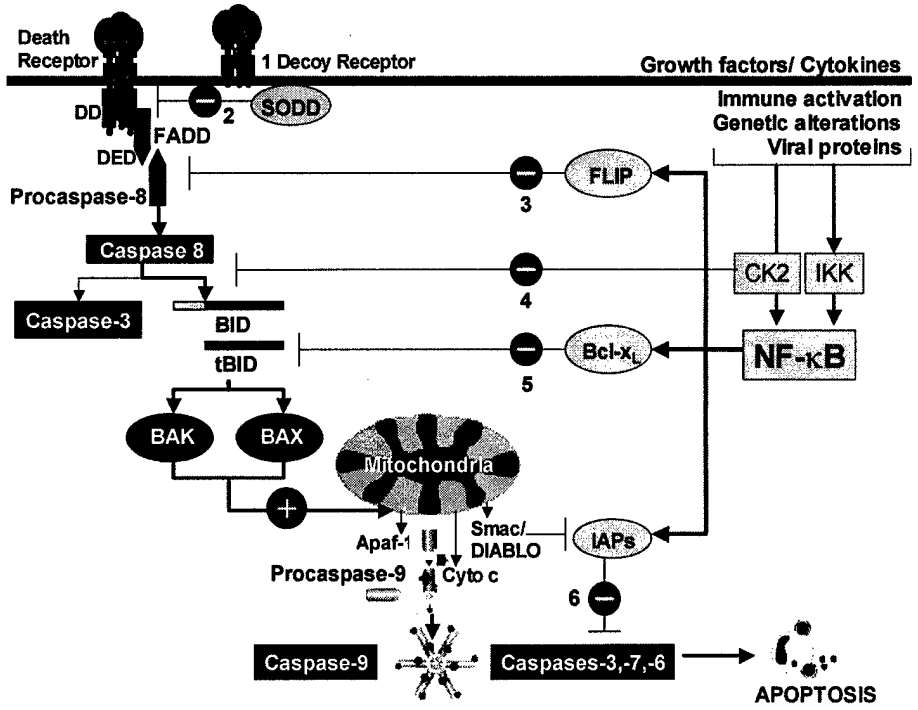

Figure 2. Schematic representation of the molecular mechanisms and regulation of death receptor-induced apoptosis.

Ligand-induced trimerization of death receptors facilitates binding of the adapter protein, FADD/MORT1, through homotypic interactions between their DDs. FADD carries a death-effector domain (DED), which interacts with the analogous DED motifs found in procaspase-8/FLICE. FADD-dependent recruitment of multiple procaspase-8 molecules to the receptor:FADD scaffold results in autocatalytic cleavage and cross-activation. Caspase-8 triggers proteolytic activation of caspase-3. Caspase-8 also cleaves and activates BID. The active truncated form of BID (tBID) translocates to the outer mitochondrial membrane, where it binds BAX or BAK. tBID-induced homoligomerization of BAX or BAK results leads to mitochondrial disruption and release of prodeath cofactors (cytochrome c, Smac/DIABLO). The interaction of cytochrome c with Apaf-1 results in a nucleotide-dependent conformational change that allows binding of procaspase-9. The formation of the procaspase-9/Apaf-1/cytochrome c complex promotes the transactivation of caspase-9. Caspase-9 activates downstream caspases (caspase-3 and caspase-7), thereby amplifying the caspase cascade and promoting apoptosis.

The death receptor-induced signaling pathway is regulated at multiple levels: (i) Decoy receptors interfere with the interaction of death ligands with their cognate death receptors. DcR1/TRAIL-R3 and DcR2/TRAIL-R4 compete with TRAIL-R1/TRAIL-R2 for Apo2L/TRAIL; DcR3 binds to CD95L and competitively inhibits the interaction of CD95 with CD95L. (ii) Silencer of death domains (SODD) inhibit the intrinsic self-aggregation of the death domain of TNFR1. (iii) The recruitment and activation of caspase-8 are inhibited by FLICE-inhibitory protein (FLIPs). (iv) Phosphorylation of BID by casein kinases (CKI and CKII) renders BID resistant to caspase-8-mediated cleavage. (v) Sequestration of tBID by the Bcl-2 homolog, Bcl-x_L, curtails its ability to promote the allosteric activation of BAX or BAK. (vi) Inhibitor-of-apoptosis proteins (IAPs) inhibit effector caspases (caspase-3, caspase-7, caspase-9, and caspase-6), until they are themselves sequestered by Smac/DIABLO. NF-κB promotes the expression of c-FLIP, Bcl-x_L, and members of the IAP family (c-IAP1, c-IAP2, XIAP). By inducing the concurrent expression of multiple antiapoptotic proteins that interrupt different steps along the death receptor-signaling pathway, NF-κB exerts a multipronged inhibition of death-receptor signals.

dependent recruitment of FADD and caspase-8 to DR4 and DR5 (Kischkel *et al.*, 2000). Regardless of the specific mechanism employed to activate caspase-8, experiments with embryonic fibroblasts from caspase-8-deficient mice confirm that caspase-8 is essential for initiation of apoptosis by CD95/Fas, TNFR1, DR3, DR4, and DR5 (Varfolomeev *et al.*, 1998).

Caspase-8-mediated activation of downstream effector caspases.

Caspase-8-mediated activation of caspase-3. In some cell types (termed 'type I'), robust activation of caspase-8 by formation of the DISC results in the direct cleavage and activation of the downstream effector caspase-3, which in turn, cleaves other caspases (such as caspase-6) and vital substrates, leading to the terminal events of apoptosis (Scaffidi *et al.*, 1998). Such cell types can undergo apoptosis via death receptor-induced activation of the caspase cascade independently of the mitochondria. However, experiments with caspase-3 knockout mice indicate that while caspase-3 may serve an important role in internucleosomal DNA fragmentation, it is not required for CD95- or TNF-induced apoptosis (Woo *et al.*, 1998).

Caspase-8-mediated cleavage of BID – mitochondrial disruption by BAX or BAK. Caspase-8 cleaves and activates BID (p22), a 'BH-3 domain only' prodeath member of the Bcl-2 family (Wang, C.Y. *et al.*, 1996; Li, J.H. *et al.*, 1998; Luo *et al.*, 1998; Gross *et al.*, 1999b; Roy and Nicholson, 2000). The active truncated form of BID (tBID; p15) translocates to the outer mitochondrial membrane, where it binds and homooligomerizes BAX or BAK, two multidomain proapoptotic members of the Bcl-2 family (Eskes *et al.*, 2000; Wei, M.C. *et al.*, 2000). tBID-induced homooligomerization of BAX or BAK results in an allosteric conformational change that leads to mitochondrial disruption and release of a cocktail of prodeath cofactors (such as cytochrome c and Smac/DIABLO) into the cytoplasm (Li, H. *et al.*, 1998; Gross *et al.*, 1999b). The interaction of the released cytochrome c with Apaf-1 results in a nucleotide-dependent conformational change that allows binding of procaspase-9 through N-terminal caspase recruitment domains (CARD) present on both molecules (Vaux, 1997). The formation of the procaspase-9/Apaf-1/cytochrome c complex (also called the 'apoptosome') promotes the transcatalytic cleavage and scaffold-mediated transactivation of caspase-9 (Srinivasula *et al.*, 1998; Stennicke *et al.*, 1999). Caspase-9 activates further downstream caspases such as caspase-3 and caspase-7, thereby amplifying the caspase cascade and promoting apoptosis (Deveraux *et al.*, 1998). Therefore, BID represents a mechanistic link between death receptor-induced activation of caspase-8 (the 'extrinsic' pathway) and the mitochondrial activation of caspases-9 and -3 (the 'intrinsic' or 'mitochondrial' pathway) (Roy and Nicholson, 2000).

Caspase-8-induced cleavage of BID is instrumental for death receptor-induced apoptosis in certain cell types (termed 'type II') which show weak DISC formation and therefore depend upon mitochondrial activation of caspase-9 to amplify the caspase cascade (Scaffidi *et al.*, 1998). Studies with BID-deficient mice indicate that BID is required for CD95-induced apoptosis in hepatocytes, but not in thymocytes or fibroblasts (Yin *et al.*, 1999). Studies of BAX-deficient, BAK-deficient, or BAX/BAK-deficient mice suggest that BAX and BAK play essential, yet mutually redundant, roles in death receptor-mediated apoptosis in hepatocytes, but are not

required for CD95-induced death of thymocytes or fibroblasts (Lindsten *et al.*, 2000; Wei *et al.*, 2001). While $BAX^{-/-}/BAK^{-/-}$ hepatocytes resist CD95-induced apoptosis, hepatocytes from either $BAX^{-/-}$ or $BAK^{-/-}$ mice remain sensitive to death receptor-induced apoptosis. In contrast, recent studies using colon carcinoma lines that have wild-type BAX and their isogenic BAX-deficient sister clones have demonstrated that BAX may be required for death receptor-induced apoptosis in cancer cells (Burns and el Deiry, 2001; Deng *et al.*, 2002; LeBlanc *et al.*, 2002; Ravi and Bedi, 2002). These reports indicate that although BAX is dispensable for apical death receptor signals, including activation of caspase-8 and cleavage of BID, it is necessary for mitochondrial activation of caspase-9 and induction of apoptosis in response to Apo2L/TRAIL, CD95/Fas, or TNF-α (LeBlanc *et al.*, 2002; Ravi and Bedi, 2002). These data suggest that the basal expression of BAK in these cells cannot substitute for BAX in mediating death receptor-induced apoptosis of tumor cells. However, upregulation of BAK expression by exposure to the chemotherapeutic agents, etoposide or irinotecan, is associated with sensitization of $BAX^{-/-}$ cancer cells to death receptor-induced apoptosis (LeBlanc *et al.*, 2002). Therefore, tBID may employ BAX or BAK for mitochondrial activation of apoptosis in a cell-type- and death signal-specific manner.

Since loss of BAX and BAK confer long-term resistance to death receptor-induced apoptosis, mitochondrial disruption appears to be critical for induction of apoptosis in type II cells. However, activation of the caspase-9/Apaf1 complex and caspase-3 is not the only mechanism by which mitochondrial disruption results in apoptosis (Cheng *et al.*, 2001). Absence of these downstream effectors provides only transient protection from tBID-induced apoptosis. Cells from $caspase\text{-}9^{-/-}$, $Apaf^{-/-}$, or $caspase\text{-}3^{-/-}$ mice remain viable for 24 h after retroviral expression of tBID, but are killed after 48 h (Cheng *et al.*, 2001). In line with these observations, cells from $caspase\text{-}9^{-/-}$ (Hakem *et al.*, 1998; Kuida *et al.*, 1998a [Q1]), $Apaf^{-/-}$ (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998), or $caspase\text{-}3^{-/-}$ (Kuida *et al.*, 1996a [Q2]; Woo *et al.*, 1998) mice remain susceptible to CD95-induced apoptosis. One possible mechanism by which tBID-mediated mitochondrial depolarization can promote death in the absence of either cyto c/Apaf-1/caspase-9 or caspase-3 may involve the activation of redundant effector caspases via release of Smac/DIABLO (second mitochondria-derived activator of caspase). Smac/DIABLO promotes caspase activation by binding and antagonizing members of the IAP (inhibitor of apoptosis) family of proteins (Du *et al.*, 2000; Verhagen *et al.*, 2000). The human IAP family comprises six members which inhibit the effector caspases-9, -3, and -7 (Deveraux *et al.*, 1997; Deveraux *et al.*, 1998; Goyal, 2001). The mitochondrial release of Smac/DIABLO into the cytoplasm via the caspase-8-BID-BAX/BAK pathway may sequester IAPs and allow activation of multiple effector caspases. The simultaneous activation of multiple redundant effector caspases may explain why deficiency of any single caspase (caspase-9 or caspase-3) is insufficient to block death receptor-induced apoptosis. It is also possible that irreversible damage to the mitochondria may itself be sufficient to induce apoptosis in a caspase-independent manner.

While these observations indicate that mitochondrial disruption via the BID-BAX/BAK pathway is essential for death receptor-induced apoptosis of certain cell types (hepatocytes, cancer cells), embryonic fibroblasts and thymocytes from BID-

deficient or BAX/BAK double-knockout (DKO) mice remain sensitive to CD95/Fas-induced apoptosis (Lindsten *et al.*, 2000; Wei *et al.*, 2001). The precise reasons for the differential requirement of the cross-talk between the extrinsic and intrinsic pathways in type I and type II cells have yet to be elucidated. These may involve biochemical differences at the receptor level and/or differences in the expression of initiator caspases and/or antiapoptotic proteins that determine the threshold that must be crossed for death receptors to activate downstream caspases.

Role of caspase-10 in death receptor-induced apoptosis.

In addition to caspase-8, death receptors can also induce recruitment and activation of the structurally related protein, caspase-10. In cells with endogenous expression of both caspase-8 and caspase-10, CD95L and Apo2L/TRAIL can recruit either protein to their DISC, where both enzymes are proteolytically activated with similar kinetics (Kischkel *et al.*, 2001). Caspase-10 recruitment and cleavage requires the adapter FADD/Mort1 and DISC assembly. Cells expressing either caspase-8 or caspase-10 can undergo ligand-induced apoptosis, indicating that each caspase can initiate apoptosis independently of the other (Kischkel *et al.*, 2001). Thus, apoptosis signaling by death receptors involves not only caspase-8 but also caspase-10, and both caspases may have equally important roles in apoptosis initiation. However, caspase-8 plays an obligatory role in death receptor-induced apoptosis of cell types that do not express caspase-10, such as many cancer cells (Kischkel *et al.*, 2001).

Role of apoptosis signal regulating kinase (ASK1) and c-Jun amino terminal kinase (JNK).

While there is overwhelming evidence that confirms the requirement of FADD/caspase-8-mediated signaling pathways in death receptor-induced apoptosis, other putative death receptor signaling pathways have also been described. Apoptosis signal regulating kinase (ASK)1 is a mammalian MAPKKK that activates SEK1 (or MKK4), which, in turn, activates stress-activated protein kinase (SAPK, also known as JNK [Q3]: c-Jun amino-terminal kinase). Overexpression of ASK1 induces apoptotic cell death, and ASK1 is activated in cells treated with TNF-α; TNF-α-induced apoptosis is inhibited by a catalytically inactive form of ASK1 (Ichijo *et al.*, 1997). These observations suggest that ASK1 may be a key element in the mechanism of stress- and cytokine-induced apoptosis. ASK1 leads to activation of c-Jun N-terminal protein kinase (JNK [Q3]). However, the role of JNK activation in TNF-α-induced apoptosis is less clear. Experiments using JNK activators or dominant negative forms of the JNK substrate c-Jun suggest a proapoptotic role of JNK in TNF-α-induced death (Verheij *et al.*, 1996). However, JNK-deficient mouse fibroblasts remain sensitive to TNF-α- or CD95L-induced apoptosis (Tournier *et al.*, 2000). Therefore JNK activation may potentiate death receptor-induced apoptosis, but is not obligatory for this process.

4. Regulation of death receptor-induced apoptosis

Death receptors play an instrumental role in the physiologic induction of apoptosis during development and tissue turnover in adult animals. However, the

unscheduled activation of death receptor-induced signals could lead to inadvertent caspase activation with devastating consequences for the organism. In order to direct the 'instructive' apoptosis of cells without sustaining uncontrolled cell death, death receptor-induced signaling is tightly regulated at multiple levels (*Figure 2*). These regulatory mechanisms are described below.

4.1 Expression of death and decoy receptors

At the most apical level, death receptor-ligand interactions may be regulated by the tissue-specific or inducible expression of death receptors or their respective ligands. TNFR1 is expressed ubiquitously, while its ligand (TNF) is expressed mainly by activated T cells and macrophages (Smith *et al.*, 1994). Likewise, CD95/Fas is widely expressed and its cell-surface expression is elevated by immune activation of lymphocytes or in response to cytokines such as interferon-γ, TNF, and CD40 ligand (CD40L) (Leithauser *et al.*, 1993; Krammer, 2000b [Q4]). Expression of CD95 ligand (CD95L) is, however, restricted to cytotoxic T cells, NK cells, and antigen presenting cells (APCs) (Li, J.H. *et al.*, 1998).

Akin to TNFR1 and CD95, the death receptors for Apo2L/TRAIL (DR4/TRAIL-R1 and DR5/TRAIL-R2) are broadly expressed in most organ systems (Golstein, 1997; Ashkenazi and Dixit, 1998). However, unlike the restricted pattern of TNF and CD95L expression in immune activated T cells and APCs, Apo2L/TRAIL mRNA is expressed constitutively in many tissues, and transcript levels increase upon stimulation in peripheral blood T cells (Screaton *et al.*, 1997a; Jeremias *et al.*, 1998; Martinez-Lorenzo *et al.*, 1998). Since several tissues constitutively express both Apo2L and its death receptors, normal cells must employ mechanisms to protect themselves from autocrine or paracrine Apo2L-DR4/DR5 interactions. One such line of defense is provided by expression of a set of decoy receptors (DcRs). DcR1 (also called TRAIL-R3, TRID, or LIT) is a glycosyl phosphatidylinositol (GPI)-anchored cell-surface protein that is structurally related to DR4 and DR5, but lacks a cytoplasmic tail (Degli-Esposti *et al.*, 1997b; Pan *et al.*, 1997b; MacFarlane *et al.*, 1997; Mongkolsapaya *et al.*, 1998; Schneider *et al.*, 1997; Sheridan *et al.*, 1997). DcR2 (also called TRAIL-R4 or TRUNDD) also resembles DR4 and DR5, but has a truncated cytoplasmic DD that is only a third as long as that of functional DDs that are capable of transducing apoptotic signals (Degli-Esposti *et al.*, 1997a; Marsters *et al.*, 1997; Pan *et al.*, 1998b). The extracellular domains of DcR1 and DcR2 compete with DR4 and DR5 for binding to Apo2L/TRAIL, but cannot initiate death signals in response to ligand engagement. Transfection of Apo2L-sensitive cells with either DcR1 or DcR2 substantially reduces their sensitivity to Apo2L-induced apoptosis. Deletion of the truncated cytoplasmic region of DcR2 does not affect its ability to protect cells from Apo2L-induced death. Enzymatic cleavage of the GPI anchor also results in the sensitization of DcR1-expressing cells to Apo2L-induced apoptosis. These observations indicate that DcR1 and DcR2 may protect normal cells from Apo2L by acting as 'decoys' that compete with TRAIL-R1/TRAIL-R2 for their shared ligand. The expression of decoy receptors provides a potential molecular basis for the relative resistance of normal cells to TRAIL/Apo2L-induced death. In support of this notion, resting peripheral T cells (that resist Apo2L) exhibit an elevation of DR5

(Screaton *et al.*, 1997a) and concomitant reduction of DcR1 levels (Mongkolsapaya *et al.*, 1998) when they acquire an Apo2L-sensitive phenotype upon activation by interleukin-2 (Martinez-Lorenzo *et al.*, 1998). However, many tumor cell lines express high levels of decoy receptors, yet remain susceptible to Apo2L-induced death (Griffith and Lynch, 1998). Therefore, it is likely that the susceptibility of cells to Apo2L-induced apoptosis must involve additional regulatory mechanisms beyond the ratio of death and decoy receptors.

A third decoy receptor, DcR3, is a secreted soluble protein the binds to CD95L (Pitti *et al.*, 1998). DcR3 competitively inhibits the interaction of CD95 with CD95L, and overexpression of DcR3 inhibits CD95-induced apoptosis (Pitti *et al.*, 1998). DcR3 mRNA is expressed in the spleen, colon, and lung. While its physiologic role remains unclear, the frequent amplification of the DcR3 gene in primary lung and colon cancers may protect tumor cells from CD95L-induced death.

4.2 Inhibition of DD signaling by silencer of death domains (SODD)

The DDs of death receptors (TNF-R1, CD95, DR3, DR4, and DR5) can self-associate and bind other DD-containing proteins. Overexpression of DD receptors may lead to ligand-independent receptor aggregation and cell death. However, cells are protected from such spontaneous ligand-independent signaling by death receptors via expression of a ~60-kDa protein termed silencer of death domains (SODD) (Jiang *et al.*, 1999). SODD associates with the DDs of TNF-R1 and inhibits the intrinsic self-aggregation of the DD (Jiang *et al.*, 1999). This inhibition is lost by triggering the release of SODD from the DD in response to cross-linkage of TNF-R1 with TNF-α. This allows ligand-dependent recruitment of adapter proteins to form an active signaling complex. However, the duration of TNF signaling is controlled by the rapid dissociation of signaling proteins from TNF-R1 and reformation of the TNF-R1-SODD complex (Jiang *et al.*, 1999). While SODD interacts with TNF-R1 and DR3, other SODD-related proteins may play a similar role in preventing ligand-independent signaling by CD95, DR4, or DR5.

4.3 Regulation of caspase-8 by FLICE-inhibitory proteins (FLIPs)

The recruitment and activation of caspases by death receptor engagement can be inhibited by FLICE-inhibitory protein (FLIP; also called I-FLICE, CASH, CLARP, MRIT, or usurpin) (Bertin *et al.*, 1997; Goltsev *et al.*, 1997; Han *et al.*, 1997; Hu *et al.*, 1997; Inohara *et al.*, 1997; Irmler *et al.*, 1997; Srinivasula *et al.*, 1997; Thome *et al.*, 1997; Rasper *et al.*, 1998; Chai, J. *et al.*, 2001). FLIP contains death-effector domains (DEDs) that bind to the DED of FADD and the prodomains of procaspases-8 and -10, thereby inhibiting their recruitment to the CD95-FADD- or TNFR1-induced activation complex (Goltsev *et al.*, 1997; Han *et al.*, 1997; Goltsev *et al.*, 1997; Han *et al.*, 1997; Irmler *et al.*, 1997; Srinivasula *et al.*, 1997). Enforced expression of v-FLIP (found in γ-herpes and pox viruses) inhibits apoptosis induced by CD95, TNFR1, DR3, and DR4/TRAIL-R1 (Thome *et al.*, 1997). The equine herpes II virus E8 protein and molluscum contagiosum MC159 and MC160 also contain DEDs homologs to those of procaspase-8, and inhibit its recruitment to the death receptor signaling complex (Bertin *et al.*, 1997).

Multiple splice variants of the human homologs of FLIP have been identified.

The longer, more abundant form, $FLIP_L$, has two DEDs and a caspase-like C-terminal domain, but lacks the catalytic cysteine and histidine residues that contribute to substrate binding. The shorter splice variant, $FLIP_S$, comprises only the two DEDs. The effects of cellular FLIPs appear to vary depending on the cellular context. Enforced expression of FLIP (the long splice variant) has an apparently paradoxical pro-apoptotic effect, possibly mediated by the aggregation of procaspase-8. However, experiments with c-FLIP-deficient mice support an antiapoptotic role of c-FLIP that is analogous to its viral counterparts. Embryonic fibroblasts from c-FLIP-deficient mice are hypersensitive to TNF-α- or CD95/Fas-induced apoptosis (Yeh *et al.*, 2000). Akin to v-FLIP, c-FLIP appears to function as a physiologic inhibitor of death receptor-induced apoptosis via homotypic DED-mediated interactions with FADD and procaspase-8 (Irmler *et al.*, 1997; Srinivasula *et al.*, 1997). c-FLIP also interacts with TRAF2 and receptor-interacting protein (RIP), which are responsible for TNFR1-induced activation of NF-κB and JNK. However, c-FLIP-deficient cells do not exhibit any change in TNF-α-induced activation of NF-κB (Yeh *et al.*, 2000). Conversely, NF-κB activation is required for TNF-α-induced expression of c-FLIP (Kreuz *et al.*, 2001). These observations indicate that NF-κB-induced expression of c-FLIP protects cells from death receptor-induced apoptosis by preventing initiation of the caspase cascade.

4.4 Regulation of BID cleavage and function

Caspase-8-induced cleavage of BID is required for mitochondrial amplification of downstream caspases in response to death receptor engagement in type II cells. Therefore, regulation of BID cleavage or activity is a mechanism of controlling death receptor-induced apoptosis in type II cells. These regulatory mechanisms are described below.

Regulation of BID phosphorylation and cleavage by casein kinases I and II.

BID is cleaved by caspase-8 at Asp 59, which resides in a large flexible loop between the second and third α helices (Li. H. *et al.*, 1998; Luo *et al.*, 1998). This cleavage site is located between the Thr and Ser residues which are phosphorylated by casein kinases I and II (CKI and CKII) (Desagher *et al.*, 2001). CKI exists as monomers of seven isoforms encoded by distinct genes (α, β, γ1, γ2, γ3, δ, and ε) (Tuazon and Traugh, 1991). CKII is an evolutionarily conserved holoenzyme composed of two catalytic α (and/or α′) subunits and two regulatory β subunits (Tuazon and Traugh, 1991). Phosphorylation of BID by CKI and CKII has been reported to render BID resistant to caspase-8-mediated cleavage (Desagher *et al.*, 2001). Conversely, a mutant of BID that cannot be phosphorylated at these residues is apparently more sensitive to caspase-8-induced cleavage and more effective than wild-type BID in promoting apoptosis. Consistent with these observations, activation of CKI and CKII reportedly delays CD95/Fas-induced apoptosis, whereas CK inhibitors potentiate death receptor-induced apoptosis (Desagher *et al.*, 2001).

While these observations suggest that the protection conferred by CKs is mediated by phosphorylation of BID, CKs may also target other proteins, such as NF-κB, that regulate the caspase-8-BID-BAX/BAK death pathway. It is also possible that other kinases, such as protein kinase C (PKC), may be involved in the phos-

phorylation of BID. Activation of PKC by phorbol esters prevents CD95-induced cleavage of BID and apoptosis, and this protective effect is reversed by inhibition of PKC (Holmstrom *et al.*, 2000). It remains to be determined whether CKs cooperate with PKC or other kinases in the phosphorylation of BID. The Ser/Thr phosphatase that dephosphorylates BID and renders it susceptible to caspase-8-mediated cleavage in response to death receptor engagement has yet to be identified.

Sequestration of BID by Bcl-x_L – competition with BAX or BAK.

BH-3 domain-only members of the Bcl-2 family, such as BID, absolutely require multidomain members of the Bcl-2 family (BAX, BAK) to induce apoptosis. Antiapoptotic members of the Bcl-2 family, such as Bcl-x_L, heterodimerize with BAX or BAK, as well as BID (Cheng *et al.*, 2001). While mutants of Bcl-x_L that cannot bind either BAX or BAK (bearing F131V or D133A substitutions) remain capable of protecting cells from death receptor-induced apoptosis, Bcl-x_L mutants that fail to bind BID (bearing G138E, R139L, and I140N substitutions) are unable to inhibit apoptosis (Cheng *et al.*, 2001). These observations support a model in which antiapoptotic Bcl-2 family members, such as Bcl-x_L, sequester tBID in stable mitochondrial complexes, thereby curtailing its ability to promote the allosteric activation of BAX or BAK. In this scenario, proapoptotic multidomain members (BAX, BAK) compete with antiapoptotic members (Bcl-2, Bcl-x_L) for binding to tBID to regulate the mitochondrial disruption and efflux required for the terminal events of apoptosis.

4.5 Inhibitor-of-apoptosis proteins (IAPs) – sequestration of Smac/DIABLO and inhibition of caspases

Caspases are directly regulated by interactions with inhibitor-of-apoptosis (IAP) proteins. At least five mammalian homologs of the baculovirus IAP have been identified. Four of these (cIAP-1, cIAP-2, XIAP, and NAIP) consist of an N-terminal domain containing multiple copies of a so-called baculovirus IAP repeat (BIR) motif (Birnbaum *et al.*, 1994), and a C-terminal zinc-containing protein–protein interaction domain (RING finger) (Lovering *et al.*, 1993). The fifth member (survivin) contains only the BIR domain. XIAP (X chromosome-linked IAP; also known as hILP), cIAP-1, and c-IAP2 (but not NAIP) directly bind and inhibit effector caspases, such as caspase-3 and caspase-7 (Deveraux *et al.*, 1997; Roy *et al.*, 1997). In addition, they also prevent activation of procaspase-9 and procaspase-6 by upstream signals (Deveraux *et al.*, 1998). XIAP inhibits caspase-3 and caspase-7 via its second BIR domain and BH2-terminal linker (Takahashi *et al.*, 1998), and prevents activation of procaspase-9 through a region containing its third BIR domain (BIR3) (Deveraux *et al.*, 1999). The BIR2 region facilitates caspase-binding, and the NH2-terminal linker directly blocks the catalytic cleft of caspase-3 and caspase-7 (Chai, J. *et al.*, 2001; Huang *et al.*, 2001; Riedl *et al.*, 2001). Consistent with its ability to inhibit multiple effector caspases, overexpression of XIAP can inhibit TNF-α-induced apoptosis (Stehlik *et al.*, 1998). While these direct interactions with caspases may be responsible for the antiapoptotic effects of IAPs, cIAP-1 and c-IAP2 also interact with the TNFR1-associated proteins, TRAF-1 and

TRAF-2, via their BIR domains (Rothe *et al.*, 1995). Therefore, although c-IAPs do not directly interact with caspase-8, it is possible their recruitment to the TNFR1 signaling complex via an interaction with TRAF-2 may regulate caspase-8 activation and/or TRAF-dependent signaling. Consistent with this scenario, expression of c-IAP1 or cIAP-2 alone was not sufficient to reduce cellular sensitivity to TNF-α-induced death; however, the expression of both c-IAP1 and c-IAP2, coupled with TRAF1 and TRAF2, suppressed TNF-α-induced apoptosis (Wang. C.Y. *et al.*, 1998).

The basal expression of mammalian IAPs varies in different cell types in response to cytokines, such as TNFα. As shall be discussed below, TNF-α-induced expression of c-IAP1, cIAP-2, and XIAP is dependent upon the NF-κB transcription factor (Chu *et al.*, 1997; Wang, C.Y. *et al.*, 1998). In these physiologic situations, IAPs may serve to keep caspases in check until they are themselves sequestered and antagonized by the mitochondrial efflux of Smac/DIABLO in response to death signals. However, the constitutively high expression of IAPs, such as survivin, in many different types of tumor cells may render such cells abnormally resistant to death receptor-induced apoptosis (Ambrosini *et al.*, 1997).

4.6 NF-κB – a master regulator of death receptor-induced apoptosis

While each of the regulatory mechanism(s) described above serves to interrupt specific steps along the death receptor-induced signaling pathway, the master regulator responsible for orchestrating the coordinated control of death receptor-induced apoptosis is NF-κB, a family of heterodimeric transcription factors (Rel proteins) that plays an important role in determining lymphocyte survival during immune, inflammatory, and stress responses (Sha *et al.*, 1995; Beg and Baltimore, 1996; Liu, Z.G. *et al.*, 1996; Van Antwerp *et al.*, 1996; Wang, C.Y. *et al.*, 1996; Attar *et al.*, 1997; Franzoso *et al.*, 1998; Alcamo *et al.*, 2001; Senftleben *et al.*, 2001b; Karin and Lin, 2002). Mammals express five Rel proteins that belong to two classes (Grimm and Baeuerle, 1993; Karin and Ben Neriah, 2000). Members of one group (RelA, c-Rel, and RelB) are synthesized as mature proteins, while the other (encoded by *NFkb1* and *NFkb2*) includes precursor proteins (p105 and p100, respectively) that undergo proteolysis to yield their mature products (p50 and p52 NF-κB proteins).

NF-κB dimers containing RelA or c-Rel are held in an inactive cytoplasmic complex with inhibitory proteins, the IκBs. Phosphorylation of IκBs at two critical serine residues (Ser^{32} and Ser^{36} in IκBα, Ser^{19} and Ser^{23} in IκBβ) in their N-terminal regulatory domain by the IκB kinase (IKK) complex targets them for rapid ubiquitin-mediated proteasomal degradation (Karin and Ben Neriah, 2000). IKK is a multisubunit protein kinase consisting of two catalytic subunits, IKKα and IKKβ, which phosphorylate IκB, and a regulatory subunit, IKKγ (also called NEMO, NF-κB essential modifier/modulator or IKKAP1), which is required for activation of IKKα/IKKβ heterodimers in response to proinflammatory cytokines, such as TNF-α and interleukin-1 (IL-1). The C-terminus of IKKγ subunit serves as a docking site for upstream signals, and the N-terminal half of IKKγ (minus the first 100 amino acids) binds to IKKβ. This results in phosphorylation of specific conserved serine residues (S177 and S181) within the T-loop (activation domain) in the cat-

alytic domain of IKKβ. Activation of the canonical NF-κB pathway involving degradation of IκB is mostly dependent on the IKKβ subunit, and is essential for innate immunity (Li *et al.*, 1999a; Delhase *et al.*, 1999; Li *et al.*, 1999b; Senftleben *et al.*, 2001b). A second pathway is involved in activation of the NF-κB dimer between RelB and p52 (Solan *et al.*, 2002). RelB is held in an inactive cytoplasmic complex by NF-κB2p100 until IKKα-dependent degradation of the IκB-like COOH-terminus of p100 allows the release and nuclear translocation of the active RelB/p52 dimer (Solan *et al.*, 2002). The activation of the RelB/p52 dimer by proteolytic processing is important for lymphoid organ development and the adaptive immune response (Senftleben *et al.*, 2001a).

In addition to the release and nuclear translocation of the dimer, transcriptional induction of target genes by NF-κB requires phosphorylation of Rel proteins by serine/threonine kinases, such as casein kinase II and Akt (Zhong *et al.*, 1997; Sizemore *et al.*, 1999; Madrid *et al.*, 2000; Wang, D. *et al.*, 2000).

Role of NF-κB in protection of cells from death receptor-induced apoptosis.

Targeted disruption of the RelA subunit of NF-κB or either IKKβ or IKKγ/NEMO results in embryonic death of mice as a result of massive hepatic (liver) apoptosis (Beg *et al.*, 1995; Li *et al.*, 1999a; Rudolph *et al.*, 2000). $RelA^{-/-}$ fibroblasts, unlike their wild-type ($RelA^{+/+}$) counterparts, exhibit a profound sensitivity to TNF-α-induced apoptosis (Beg and Baltimore, 1996). Likewise, $IKK\beta^{-/-}$ fibroblasts, or cells stably transfected with phosphorylation mutants of IκBα, fail to activate NF-κB and display increased sensitivity to TNF-α-induced death (Van Antwerp *et al.*, 1996; Li *et al.*, 1999b; Senftleben *et al.*, 2001b). These observations demonstrate an important role of NF-κB in protecting cells from death receptor-induced apoptosis.

Engagement of TNFR1 by TNF leads to the recruitment of the adapter protein TRADD to the clustered DDs of the trimerized receptors. TRADD, in turn, serves as a platform for the docking of multiple signaling molecules to the activated receptor complex. As discussed earlier, TNF-induced apoptosis is triggered by recruitment of the adapter molecule FADD to the TNFR1-TRADD complex. Therefore, the apoptotic signaling pathways triggered by different members of the death receptor family (TNFR1, CD95/Fas, and TRAIL-R1/R-2) are all initiated by ligand-induced recruitment of FADD and FADD-mediated activation of caspase-8. While they share a common death-signaling pathway, these receptors exhibit a differential ability to activate NF-κB. While FasL is unable to activate NF-κB, TNF-α induces activation of the transcription factors, NF-κB and JNK/AP1, via recruitment of receptor-interacting protein (RIP) and TNFR-associated factor-2 (TRAF-2) to the receptor complex. TRAF-2 and RIP activate the NF-κB-inducing kinase (NIK), which, in turn, activates the IκB kinase (IKK) and IKKβ-dependent activation of NF-κB (Malinin *et al.*, 1997; Kelliher *et al.*, 1998; Scheidereit, 1998). TRAF2 and RIP also stimulate JNK/AP-1 via activation of apoptosis signal regulating kinase (ASK)1 (Nishitoh *et al.*, 1998). Cells from TRAF2 gene knockout mice or transgenic mice expressing a dominant negative TRAF2 mutant fail to activate JNK in response to TNF, but have only slight defects in TNF-induced activation of NF-κB (Lee *et al.*, 1997; Yeh *et al.*, 1997). In contrast, RIP-deficient cells remain capa-

ble of activating JNK but lack the ability to activate NF-κB in response to TNF (Kelliher *et al.*, 1998). Therefore, RIP is essential for TNF-induced activation of NF-κB, while TRAF2 is required for signaling the activation of JNK. In addition to inducing expression of diverse proinflammatory and immunomodulatory genes, NF-κB promotes the expression of genes that protect cells from TNF-induced apoptosis (discussed below). Since the proapoptotic activity of TNF-α is opposed by the concurrent expression of antiapoptotic NF-κB target genes, the ability of TNF-α to induce apoptosis requires the inhibition of NF-κB. The differential ability to activate NF-κB may explain why TNF-α, unlike FasL, rarely triggers apoptosis unless new protein synthesis is simultaneously blocked (Baud and Karin, 2001).

In addition to protecting cells from the latent death-signaling arm of TNFR1, TNF-α-induced activation of NF-κB promotes the expression of a host of proinflammatory and immunomodulatory genes that mediate the biologic function of this cytokine. In the absence of the protection conferred by NF-κB, TNF-α loses its native function in the immune response and, instead, acquires a proapoptotic role. The mid-gestational lethality of $RelA^{-/-}$, $IKK\beta^{-/-}$, or $IKK\gamma^{-/-}$ mice results from the extensive hepatocyte apoptosis induced by the production of TNF-α by hematopoietic progenitors that are resident in the fetal liver. The massive liver apoptosis resulting from embryonic deficiency of RelA is completely reversed by the concurrent deficiency of TNFR1 or TNF-α in DKO mice (Doi *et al.*, 1999; Rosenfeld *et al.*, 2000; Alcamo *et al.*, 2001). NF-κB also protects lymphoid cells from death receptor-induced apoptosis during the immune response (Van Parijs *et al.*, 1996a). Activation of NF-κB by co-stimulation of lymphocytes mediates cell survival and clonal proliferation, while inhibition of NF-κB by IκB mutants promotes activation-induced apoptosis of T cells, and loss of $CD8^+$ T cells in the thymus. As shall be discussed later, NF-κB-mediated protection of cells from death receptor-induced apoptosis plays an instrumental role in regulating the immune response.

Molecular mechanisms by which NF-κB regulates death receptor-induced apoptosis.

NF-κB is a critical determinant of the expression of genes that modulate death receptor-induced apoptosis. NF-κB promotes the expression of a number of survival factors, including the caspase-8/FLICE inhibitor (c-FLIP), members of the inhibitor of apoptosis (IAP) family (c-IAP1, c-IAP2, XIAP), TNFR-associated factors (TRAF1 and TRAF2), and the Bcl-2 homologs, A1 (also known as Bfl-1) and Bcl-x_L. As discussed above, these proteins serve to interrupt different steps along the death receptor-signaling pathway. By inducing the concurrent expression of multiple antiapoptotic proteins, NF-κB exerts a multipronged inhibition of death-receptor signals (*Figure 2*).

c-FLIP, is an NF-κB-inducible protein (encoded by *Cflar*) that prevents death receptor-induced activation of the initiator procaspase-8 (Irmler *et al.*, 1997; Yeh *et al.*, 2000). NF-κB activation is required for TNF-α-induced expression of c-FLIP (Kreuz *et al.*, 2001). Although TNF-α-induced induction of c-FLIP is repressed by a degradation-resistant mutant of IκBα, c-FLIP can still be induced by TNF-α in $RelA^{-/-}$ cells (Yeh *et al.*, 2000). Therefore, the identity of the NF-κB dimer responsible for promoting c-FLIP expression remains unknown.

The promoter of the *cIap2* gene contains two functional κB sites (Hong *et al.*, 2000). Induction of *c-Iap2* by TNF-α is blocked by introduction of a phosphorylation mutant form of IκBα that resists IKK-induced degradation (Wang, C.Y. *et al.*, 1998). Akin to c-IAP2, c-IAP1 and XIAP are also NF-κB-induced proteins which block the activation of caspases (-3, -7, and -9) by death receptors (Liston *et al.*, 1996).

c-IAPs cannot directly interact with procaspase-8, and expression of either protein alone is not sufficient to protect cells from TNF-α-induced death (Wang, C.Y. *et al.*, 1998). Therefore, the recruitment of c-IAPs to the TNFR1 signaling complex via interactions with TRAF2 may be required for inhibition of caspase-8 and proximal blockade of the death signal (Shu *et al.*, 1996; Wang, C.Y. *et al.*, 1998). TRAF1 and TRAF2 are also NF-κB-inducible genes that, along with c-IAP1 and c-IAP2, suppress TNF-α-induced activation of caspase-8. Since TRAF2 also serves as an adapter that augments TNF-α-induced activation of NF-κB, TRAF2 and NF-κB may participate in a positive feedback loop to prevent TNF-α-induced apoptosis. Consistent with this notion, cells from $TRAF2^{-/-}$ mice are partially defective in TNF-α-induced NF-κB activation and exhibit exaggerated sensitivity to TNF-α-induced apoptosis (Lee *et al.*, 1997; Yeh *et al.*, 1997).

The prototypic antiapoptotic member of the Bcl-2 family, Bcl-x_L, contains a κB DNA site (TTTACTGCCC; 298/+22) in its promoter (Chen, C. *et al.*, 2000). The Rel-dependent induction of Bcl-x_L in response to TNF-α is sufficient to protect cells carrying a degradation-resistant form of IκB from TNF-α-induced death (Chen, C. *et al.*, 2000). Likewise, NF-κB-dependent expression of Bcl-x_L in response to co-stimulatory signals (CD40-CD40L or CD28-B7 interactions) serves to protects B or T cells from CD95/Fas- or Apo2L/TRAIL-induced apoptosis (Chen, C. *et al.*, 2000; Ravi *et al.*, 2001) .

Bfl-1/ A1 is a hematopoietic-specific Bcl-2 homolog which contains a functional κB site in its promoter and is induced in an NF-κB-dependent fashion in response to TNF-α (Zong *et al.*, 1999). Overexpression of A1 partially protects Rel-deficient cells from TNF-α-induced apoptosis (Zong *et al.*, 1999). Since $A1^{-/-}$ mice exhibit only increased neutrophil apoptosis, it is likely that A1 is dispensable for the antiapoptotic function of NF-κB in all other tissue types (Hamasaki *et al.*, 1998).

NF-κB may also protect cells from death receptor-induced apoptosis by attenuating expression of the proapoptotic protein, BAX (Bentires-Alj *et al.*, 2001). In certain cell types, inhibition of NF-κB by a degradation-resistant mutant of IκBα results in increased *Bax* promoter activity and expression of BAX. Although the *Bax* promoter has a κB site that binds Rel proteins, it is not required for NF-κB-mediated inhibition of BAX expression. Therefore, NF-κB may increase BAX expression via an indirect mechanism. One possibility is that NF-κB may repress stimulation of the *Bax* promoter by interfering with the function of the *p53* tumor suppressor gene. While the precise molecular mechanism remains unclear, the reduced expression of BAX may contribute to the resistance of cells with constitutive activation of NF-κB to death receptor-induced apoptosis. As discussed earlier, targeted loss of the *Bax* gene renders cancer cells resistant to CD95L, TNF-α, and Apo2L/ TRAIL-induced apoptosis Deng *et al.*, 2002; LeBlanc *et al.*, 2002; Ravi and Bedi, 2002). Since NF-κB is constitutively activated by diverse genetic aberrations

in human cancers, NF-κB-mediated repression of BAX may play a role in protecting tumor cells from death receptor-ligand interactions (Ravi and Bedi, 2002).

Finally, NF-κB also induces expression of a JNK inhibitor (De Smaele *et al.*, 2001; Javelaud and Besancon, 2001; Tang *et al.*, 2001a). TNF-α-induced activation of JNK occurs transiently in normal cells but is increased and prolonged in IKKβ- or RelA-deficient cells (Tang *et al.*, 2001a). Although the identity of the NF-κB-induced JNK inhibitor remains unclear, the identified candidates include XIAP and the GADD45β protein (De Smaele *et al.*, 2001; Tang *et al.*, 2001a). Ectopic expression of GADD45β abrogates TNF-α-induced activation of JNK and rescues $RelA^{-/-}$ cells from TNF-α-induced apoptosis. Although the suppression of JNK may contribute to the antiapoptotic function of NF-κB, JNK is not an essential mediator of death receptor-induced apoptosis.

These observations suggest that NF-κB inhibits death receptor-induced apoptosis by concomitant induction of multiple survival genes as well as repression or inactivation of proapoptotic genes.

4.7 The dynamic balance between death receptors and apoptosis-inhibitors – a tug-of-war that determines cell fate

The activation of caspases by death receptor-induced signals is held in check by the antiapoptotic proteins described above. While this serves to prevent unscheduled or uncontrolled cell death, the induction of apoptosis in response to physiologic death signals requires mechanisms to circumvent or overcome these antiapoptotic proteins. Without such mechanisms, the failure of death receptor-induced apoptosis would disrupt homeostasis and result in immune and neoplastic disorders. The dynamic balance between the antagonistic functions of death receptors and antiapoptotic proteins ensures that death receptor-induced apoptosis is allowed to proceed in a signal-dependent, scheduled, and controlled fashion. The molecular mechanisms by which this balance is tipped in favor of instructive apoptosis involves signal-induced expression of death receptors and caspase-mediated proteolysis of many of the key proteins that inhibit death receptor-induced apoptosis.

Expression of death receptors.

Cell-surface expression of CD95/Fas is elevated by immune activation of lymphocytes or in response to cytokines such as interferon-γ, TNF, and CD40 ligand (CD40L) (Leithauser *et al.*, 1993; Krammer, 2000a)[Q4]. Likewise, immune activation of lymphocytes results in an elevation of DR5 (Screaton *et al.*, 1997a) and concomitant reduction of DcR1 levels (Mongkolsapaya *et al.*, 1998). Since immune activation and clonal lymphoid expansion must be followed by cellular demise to preserve homeostasis, the immune system must employ molecular mechanisms to couple immune activation (and expression of survival proteins) with the induction of death receptors that mediate the decay of the immune response. One mechanism by which the immune system accomplishes this task is by employing NF-κB. While activation of NF-κB by costimulation of lymphocytes induces death receptors (CD95, DR5) (Ravi *et al.*, 2001; Zheng *et al.*, 2001), the activity of the induced receptors is held in check by the concurrent NF-κB-mediated induction of antiapoptotic proteins (described earlier) (Ravi *et al.*, 2001). Engagement of the

induced receptors by their cognate ligands (expressed by activated T cells or DCs) may become capable of inducing apoptosis following decay of NF-κB activity at the termination of immune stimulation or by caspase-mediated inactivation of NF-κB (see below).

Caspase-mediated cleavage of antiapoptotic proteins.

Once death receptors are induced and engaged, the activation of caspases is amplified by caspase-induced proteolytic cleavage of several key antiapoptotic proteins, including NF-κB and NF-κB-induced survival proteins. The proteins targeted by caspases and the functional effects of such cleavage are described below.

Inactivation of NF-κB by caspase-mediated proteolysis – loss of survival gene expression. The proteins responsible for mediating TNF-α-induced activation of NF-κB are themselves substrates of caspases. Caspase-8-mediated cleavage of RIP at Asp^{32} destroys its ability to activate IKK (Lin *et al.*, 1999). In addition to inhibiting NF-κB activation, the NH_2-terminal-deficient fragment (RIPc) generated by such cleavage promotes the assembly of TNFR1-TRADD-FADD complex and potentiates TNF-α-induced apoptosis (Lin *et al.*, 1999). Proteolysis of TRAF1 and TRAF2 also results in increased sensitivity to death receptor-induced apoptosis (Duckett and Thompson, 1997; Arch *et al.*, 2000; Leo *et al.*, 2001). Caspase-8-mediated cleavage of TRAF1 at Asp^{163} during TNF-α- or CD95L-induced apoptosis generates a COOH-terminal fragment that inhibits TRAF2- or TNFR1-mediated activation of NF-κB (Leo *et al.*, 2001). Since the truncated protein contains a TRAF domain, it may act as a dominant negative inhibitor of interactions of TRAF1 with either TRAF2 or c-IAPs (Schwenzer *et al.*, 1999).

IKKβ, the catalytic subunit responsible for the canonical pathway of NF-κB activation, is itself inactivated by caspase-3-mediated proteolysis (at Asp^{78}, Asp^{214}, Asp^{373}, and Asp^{546}) during TNF-α- or CD95-induced apoptosis (Tang *et al.*, 2001b). Expression of the IKKβ(1-546) fragment inhibits endogenous IKK and sensitizes cells to TNF-α-induced apoptosis (Tang *et al.*, 2001b). Conversely, overexpression of a caspase-resistant mutant of IKKβ promotes the sustained activation of NF-κB and prevents TNF-α-induced apoptosis. Therefore, caspase-mediated cleavage of IKKβ may be a mechanism by which caspases terminate the activation of NF-κB and remove the key obstacle to their own activity. Caspases may also achieve this end by proteolytic removal of the NH_2-terminal domain (containing the Ser^{32} and Ser^{36} phosphorylation residues) of IκBα, thereby generating a IκB fragment that is resistant to TNF-α-induced degradation and functions as a super-repressor of NF-κB activation (Barkett *et al.*, 1997; Reuther and Baldwin, 1999).

While caspases may prevent activation of NF-κB via proteolytic inactivation of the upstream signals (described above), ligation of death receptors can directly induce caspase-mediated cleavage of RelA (Ravi *et al.*, 1998a). The truncation of the transactivation domain generates a transcriptionally inactive dominant-negative fragment of RelA that serves as an efficient proapoptotic feedback mechanism between caspase activation and NF-κB inactivation (Levkau *et al.*, 1999).

These observations suggest that the protection conferred by NF-κB against death receptor-induced apoptosis may be eliminated by caspase-mediated prote-

olysis of the RIP/TRAF-IKK-IκBα-RelA pathway, thereby tilting the dynamic balance between death receptors and NF-κB-induced survival proteins in favor of cell death. The direct cleavage of RelA ensures the irreversible loss of NF-κB activity, resulting in the rapid amplification of caspase activity and inevitable cell death.

Caspase-mediated cleavage of Bcl-2, Bcl-x_L, and IAPs – amplification of caspase activity. Many of the key antiapoptotic proteins that inhibit caspases are themselves targets of caspases. Antiapoptotic members of the Bcl-2 family (Bcl-2 and Bcl-x_L) compete with proapoptotic multidomain members (BAX, BAK) for binding to tBID. As such, Bcl-2 and Bcl-x_L inhibit tBID-mediated activation of BAX/BAK, thereby limiting mitochondrial disruption. However, both Bcl-2 and Bcl-x_L are themselves substrates for caspases. The loop domain of Bcl-2 is cleaved at Asp^{34} by caspase-3 *in vitro*, in cells overexpressing caspase-3, and during induction of apoptosis by death receptors (CD95/Fas, TRAIL-R1/R2) (Cheng *et al.*, 1997; Ravi *et al.*, 2001). Death receptor-induced caspase-mediated proteolytic cleavage of Bcl-2 inactivates its survival function by removal of the BH4 domain. The carboxyl-terminal Bcl-2 cleavage product, which retains the BH3 homology and transmembrane regions, behaves as a BAX-like death effector and potentiates apoptosis (Cheng *et al.*, 1997). Cleavage of Bcl-2 contributes to amplification of the caspase cascade, and cleavage-resistant mutants of Bcl-2 confer increased protection against apoptosis. Akin to Bcl-2, Bcl-x_L is cleaved by caspases during induction of apoptosis by diverse stressful stimuli (Clem *et al.*, 1998; Fujita *et al.*, 1998). Likewise, proteolytic cleavage converts Bcl-x_L into two prodeath fragments. However, it is not yet known whether Bcl-x_L is cleaved during death receptor-induced apoptosis or whether cleavage-resistant mutants of Bcl-x_L offer better protection against death receptor-induced apoptosis. Akin to Bcl-2 family members, both c-IAP1 and XIAP are also caspase substrates (Deveraux *et al.*, 1999; Clem *et al.*, 2001). Overexpression of the caspase-induced cleavage product of c-IAP1 induces apoptosis (Clem *et al.*, 2001). Likewise, caspase-induced cleavage of XIAP at Asp^{24} generates a COOH-terminal fragment that potentiates CD95-induced apoptosis (Deveraux *et al.*, 1999).

These observations suggest that caspase-induced proteolysis of these survival proteins may serve to amplify rapidly the caspase cascade, thereby potentiating death receptor-induced apoptosis.

5 Role of death receptor-induced apoptosis in the immune system

5.1 Regulation of the immune response

Apoptosis plays an essential role in the immune system. The molecular regulation of cell survival is a fundamental determinant of lymphocyte maturation, receptor repertoire selection, homeostasis, and the cellular response to stressful stimuli, such as DNA damage (Krammer, 2000 [Q4]). Increased apoptosis is involved in the pathogenesis of diverse immune disorders. Conversely, genetic aberrations that render cells incapable of executing their suicide program result in autoimmune disorders and tumorigenesis. The physiologic role played by death receptors in the immune response is summarized below.

Activation of immune and inflammatory responses.

TNF-α is produced by activated T cells and macrophages. TNF-α or TNFR knockout mice exhibit a deficient inflammatory response to bacterial endotoxin and have an exaggerated susceptibility to microbial infections (Smith *et al.*, 1994). While these findings demonstrate a role of TNF-α in immune and inflammatory responses, TNF-α can also induce apoptosis in certain cell types or contexts. The molecular mechanisms that underlie these divergent effects of TNF-α have been described earlier.

Maintenance of lymphocyte homeostasis.

The elimination of lymphocytes during development, receptor repertoire selection, and the decay phase of the immune response involve death receptor-ligand interactions.

Deletion of thymocytes and peripheral T cells. Pre-T cells undergo maturation and rearrangement of T-cell antigen receptor (TCR) genes in the thymus. T cells that fail to undergo TCR rearrangement are incapable of stimulation by self major histocompatibility (MHC) antigen-peptide complexes and suffer death by neglect. Since this process is impaired in transgenic mice carrying a dominant-negative form of the adapter FADD, death receptors may be involved in the induction of apoptosis at this pre-TCR stage of development (Newton *et al.*, 2000). Thymocytes that survive pre-TCR selection mature into $CD4^+CD8^+$ T cells and undergo further positive and negative selection depending on the affinity of their TCRs for self MHC antigens. T cells with high affinity for self MHC molecules and peptide are eliminated, and the surviving mature $CD4^+$ MHC class-II-restricted and $CD8^+$ MHC class-I-restricted T cells leave the thymus and enter the peripheral T-cell pool in secondary lymphoid organs. The negative and positive selection that occurs during T-cell development is instrumental for self-MHC restriction and prevention of autoimmunity. Studies of T-cell receptor transgenic mice indicate that CD95 is involved with peripheral, but not thymic, deletion of T cells (Singer and Abbas, 1994; Van Parijs *et al.*, 1996b [Q5]). Although the TCR repertoire is not altered in mice that have genetic deficiencies of the CD95-CD95L system (*lpr* and *gld* mice), CD95-induced apoptosis may be involved in the negative selection of thymocytes that encounter high antigen concentrations (Kishimoto *et al.*, 1998; Newton *et al.*, 1998). The role of other death receptor-ligand interactions (such as DR4/DR5-Apo2L/TRAIL) in thymic deletion is as yet unknown.

Peripheral T cells are activated by cross-linkage of the TCR by the MHC-peptide complex together with costimulatory signals delivered by engagement of the CD28 receptor on the T cell by members of the B7 family expressed on APCs. Costimulation of T cells protects cells from TCR-induced apoptosis via activation of NF-κB- and NF-κB-dependent expression of antiapoptotic proteins, such as Bcl-x_L and c-FLIP (Boise *et al.*, 1995; Khoshnan *et al.*, 2000). TCR ligation in the absence of costimulatory signals result in T-cell apoptosis via mechanism(s) that do not require CD95-CD95L interactions (Van Parijs *et al.*, 1996b). Following clonal expansion, antigen-specific T cells acquire an apoptosis-sensitive phenotype that enables their eventual demise during the decay phase of the immune response. This form of instructive apoptosis (termed 'activation-induced cell

death' [AICD]) is required to return the expanded pool of cells to baseline levels. Unlike TCR-induced death of resting T cells, AICD is mediated by CD95–CD95L interactions at its initiation (Singer and Abbas, 1994; Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995) and the TNF-α-TNFR system at a later phase of execution (Zheng *et al.*, 1995). However, AICD may also involve other death receptors-ligands (such as Apo2L/TRAIL) or death-inducing mechanism(s) that activate caspases and/or disrupt mitochondria independent of death receptor–ligand interactions (Martinez-Lorenzo *et al.*, 1998; Hildeman *et al.*, 1999; Spaner *et al.*, 1999).

Instructive apoptosis of B cells. B cells express cell-membrane receptors (antibodies) with single antigen specificity. The specificity of the immune response is achieved by antigen binding and selection of pre-existing clones of antigen-specific lymphocytes. Autoreactive B cells are deleted in the bone marrow. Mature B cells that populate secondary lymphoid organs undergo somatic hypermutation with elimination of low-affinity or autoreactive B-cell mutants (Lam and Rajewsky, 1998). Although B cells do not express CD95L, they express CD95 and may be susceptible to elimination by CD95L expressed by T cells and APCs (Scott *et al.*, 1996). It is possible that such fratricidal death receptor–ligand interactions may play a role in elimination of low-affinity or autoreactive B cells.

Ligation of the antigen-specific B-cell receptor (BCR) alone is an insufficient signal to activate a B cell and instead results in apoptosis or functional elimination. Activation of a B cell to effector function requires delivery of a second signal by activated helper T cells. Activation of the $CD4^+$ T cell (by engagement of the TCR and ligation of CD28) induces expression by the T cell of a 39-kDa glycoprotein called gp39 or CD40 ligand (CD40L), whose receptor, CD40, is present on B cells (Foy *et al.*, 1994; Renshaw *et al.*, 1994; Xu *et al.*, 1994; Foy *et al.*, 1996). In addition to CD40-CD40L, two other receptor-ligand pairs within the TNF-R/TNF superfamilies regulate B-cell survival. Ligation of the B-cell receptors, TACI and BCMA, by BLyS (TALL-1, THANK, BAFF, zTNF4) and APRIL, respectively, promotes B-cell survival, proliferation, and immunoglobulin production (Mackay *et al.*, 1999; Moore *et al.*, 1999; Mukhopadhyay *et al.*, 1999; Khare *et al.*, 2000; Yan *et al.*, 2000). There are striking similarities between the BLyS-TACI and CD40L-CD40 systems; both ligands are TNF family members expressed on activated T cells and DCs, and both receptors are TNFR homologs expressed on B cells. Upon engagement by their respective ligands, both TACI and CD40 induce activation of NF-κB- and NF-κB-dependent expression of survival proteins (Bcl-x_L) that rescue B cells from BCR- or CD95-induced apoptosis (Lagresle *et al.*, 1996). In the absence of such protection, cell death may be triggered via CD95–CD95L interactions as well as CD95-independent BCR-induced signals that lead to mitochondrial disruption and caspase activation (Berard *et al.*, 1999; Craxton *et al.*, 1999).

After clonal expansion, antigen-reactive lymphocytes are titrated down until the lymphoid cell pool is restored to its basal level. The decay of the immune response is achieved by elimination of lymphocytes via instructive apoptosis involving death receptor/ligand systems. While CD40 engagement is required for B-cell proliferation, it also promotes the expression of CD95 on activated B cells.

Activated APCs and T cells synthesize the adversarial death ligands that kill B cells at the end of an immune response (Scott *et al.*, 1996).

Induction of cell death by cytotoxic T cells and NK cells.

Mature $CD8^+$ T cells (cytotoxic T lymphocytes [CTLs]) and natural killer (NK) cells are effectors of innate and adaptive immune responses to intracellular pathogens, cancer cells, or transplanted tissues. CTLs and NK cells induce apoptosis of these targets by two major mechanisms. One mechanism involves ligation of CD95 on target cells by FasL expressed on CTLs (Li, J.H. *et al.*, 1998). The second mechanism involves calcium-dependent exocytosis of the CTL-derived granule proteins, perforin and granzymes (Heusel *et al.*, 1994; Shresta *et al.*, 1995). Perforin facilitates the delivery of granzyme B into target cells via an as yet obscure mechanism that does not require plasma membrane pore formation (Shi *et al.*, 1997; Metkar *et al.*, 2002). Granzyme B, the prototypic member of this family of serine proteases, induces cleavage and activation of multiple caspases, including caspase-3, -6, -7, -8, -9, and -10 (MacDonald *et al.*, 1999). Granzyme B also cleaves BID at a site distinct from that targeted by caspase-8 (Alimonti *et al.*, 2001). Akin to tBID (generated by caspase-8), the truncated BID generated by granzyme B (gtBID) translocates to the mitochondrial membrane and promotes the release of mitochondrial death factors via BAX or BAK (Heibein *et al.*, 2000; Alimonti *et al.*, 2001; Wang, N.S. *et al.*, 2001). Since granzyme B and CD95L can both activate the BID-BAX/BAK death-signaling pathway, they provide independent mechanisms of inducing target cell apoptosis. Accordingly, cells deficient in CD95 or overexpressing c-FLIP remain susceptible to CTL-induced death (Kataoka *et al.*, 1998). It will be important to determine whether interruption of a distal step of the death-signaling pathway shared by CD95L and granzyme B (such as loss of BAX/BAK) reduces CTL-induced death of type II target cells that require cross-talk between the extrinsic and intrinsic pathways to undergo apoptosis. Such genetic impediments to CTL-induced death may be an important mechanism by which tumor cells evade immune surveillance.

Establishment of zones of immune privilege.

Immune-privileged sites such as the eye, brain, and the testes may evade damage by constitutively expressing CD95L to counterattack and eliminate CD95-expressing infiltrating lymphocytes (Green and Ferguson, 2001). Some reports suggest that expression of CD95L by certain types of cancer cells may protect such tumors from immune surveillance. While ectopic expression of FasL by gene transfer can confer immune privilege on some tissues, it can also induce a granulocytic infiltrate and increased rejection in tissue transplants. The role of CD95L–CD95 interactions in the creation of zones of immune privilege in tumors or tissue allografts *in vivo* remains debatable (Green and Ferguson, 2001).

5.2 Disorders of the immune system resulting from deregulation of death receptor-induced apoptosis

The functional importance of the normal physiologic role of death receptors in the immune system is evident from the occurrence of various disorders resulting from dysfunctional death receptors/death ligands or their signaling pathways.

Autoimmune disorders from genetic defects in CD95-induced apoptosis.

Mutations that result in defects of the CD95-CD95L system result in immune disorders that feature lymphadenopathy and autoimmunity. The recessive *lpr* (lymphoproliferation) mutation results in a splicing defect that reduces expression of CD95, while the lpr^{cg} is a point mutation in the DD of CD95 that makes it incapable of transducing a death signal (Nagata, 1997). The *gld* (generalized lymphoproliferative disease) mutation in the carboxy domain of CD95L interferes with its interaction with CD95 (Nagata, 1997). The immune disorders manifest in mice carrying these mutations result from the loss of CD95-CD95L-induced apoptosis. Defects of the CD95-CD95L system also result in similar immune disorders in humans (Fisher *et al.*, 1995; Rieux-Laucat *et al.*, 1995; Nagata, 1998). Children with type Ia autoimmune lymphoproliferative syndrome (ALPS; Canale Smith syndrome) frequently have mutations in the DD of CD95 and exhibit lymphadenopathy, aberrant accumulation of T cells, and autoimmunity. While type Ib ALPS is associated with defects in CD95L, type II ALPS involves defects in CD95 signaling, such as mutations in caspase-10 (Wang, H.G. *et al.*, 1999).

Augmentation of death receptor-induced apoptosis in AIDS.

Autoimmune [Q6] deficiency syndrome (AIDS) is characterized by an excessive depletion of $CD4^+$ T helper cells via apoptosis. Several different mechanisms underlie the increased apoptosis of $CD4^+$ T cells in response to infection with the human immunodeficiency virus (HIV). HIV-encoded gene products (such as HIV-1 Tat) increase expression of CD95L, which promotes TCR-induced CD95-mediated apoptosis as well as fratricidal deletion of uninfected T cells (Debatin *et al.*, 1994; Finkel *et al.*, 1995; Li, C.J. *et al.*, 1995; Westendorp *et al.*, 1995). T cells from HIV-infected individuals also show increased sensitivity to Apo2L/TRAIL (Jeremias *et al.*, 1998). In addition to CD95L- and Apo2L/TRAIL-induced apoptosis, HIV-binding of CD4 and the chemokine receptor CXCR4 also contributes to the rapid depletion of $CD4^+$ T cells in AIDS (Gougeon and Montagnier, 1999).

6 Role of death receptor-induced apoptosis in development

Physiologic cell death is essential for animal development, maintenance of adult tissue homeostasis, tissue remodeling and regeneration, and elimination of cells with genetic or stochastic developmental errors (Meier *et al.*, 2000). The apoptotic culling of overproduced, unnecessary, misplaced, or damaged cells occurs via different mechanisms. Such cell death may occur autonomously via withdrawal of the appropriate trophic survival signals or transcriptional induction of genes that promote apoptosis (Raff, 1992). The survival of different cell types depends on specific survival signals within their specialized microenvironments. These survival signals include cytokines, hormones, interactions with neighboring cells, and the extracellular matrix, as well as more specialized signals such as neuronal synaptic connections or productive assembly of an appropriate immune receptor. Withdrawal of such survival signals results in cell death by default or neglect. Interference with the autonomous deletion of cells is manifested by not only developmental abnormalities, but also a wide variety of adult

pathologies, such as neoplastic disorders. Spontaneous or induced mutations in the mitochondrial cell death machinery manifest as phenotypic defects that are especially evident in the immune and nervous systems. Mice lacking caspase-9, Apaf-1, or caspase-3 exhibit gross neuronal hyperproliferation and abnormalities in brain development (Kuida *et al.*, 1996b[Q2]; Cecconi *et al.*, 1998; Hakem *et al.*, 1998; Kuida *et al.*, 1998a; Kuida *et al.*, 1998b[Q1]; Yoshida *et al.*, 1998). Transgenic mice with tissue-specific overexpression of Bcl-2 or Bcl-x_L exhibit extended cell survival and pathologic cellular accumulation in the targeted tissues (Strasser *et al.*, 2000). Conversely, mice lacking Bcl-2 show premature demise of mature lymphocytes, while Bcl-x-deficiency results in increased neuronal apoptosis and embryonic lethality (Motoyama *et al.*, 1995; Middleton *et al.*, 2000).

In addition to death by neglect from loss of trophic survival signals, developmental cell death may also require activation of death receptor-induced caspase-8-mediated signaling pathways. The principal effects of the loss of TNF, CD95 or their cognate ligands are on the immune system (Yeh *et al.*, 1999). However, death receptor-induced apoptosis may also be important in other systems. Apoptosis of spinal motor neurons in response to trophic factor withdrawal is inhibited by an antagonistic anti-CD95 antibody (Raoul *et al.*, 1999). Caspase-$8^{-/-}$ mice suffer embryonic lethality after day 11.5, with poorly developed heart musculature and abnormal accumulation of erythrocytes in the liver, lung, lens, and mesenchyma (Varfolomeev *et al.*, 1998). $FADD^{-/-}$ embryos die at the same stage of development with similar cardiac abnormalities, and $FADD^{-/-}$ T cells exhibit impaired antigen-induced proliferation (Zhang *et al.*, 1998). The absence of obvious developmental defects in most tissues in mice lacking specific TNF-R or TNF family members, and the focal failure of specific tissues in $FADD^{-/-}$ or caspase-$8^{-/-}$ mice are indicative of functional overlap and redundancy within the TNF-R/TNF-family and between the mitochondrial and death receptor-induced signaling pathways.

7 Death receptor-induced apoptosis of tumor cells

7.1 Role of death receptors/ligands in tumor surveillance

Death receptor-ligand interactions may serve a critical physiologic function in tumor surveillance (Kashii *et al.*, 1999). NK cells play a pivotal role in the control of tumor metastasis (Talmadge *et al.*, 1980; Karre *et al.*, 1986). Freshly isolated murine liver NK cells, but not natural killer T cells or ordinary T cells, constitutively express cell-surface Apo2L/TRAIL, which, together with perforin and Fas ligand (FasL), mediate NK cell–dependent suppression of experimental liver metastasis of tumor cells (Takeda *et al.*, 2001). Administration of neutralizing monoclonal antibodies against either Apo2L/TRAIL or FasL significantly increases hepatic metastases of several tumor cell lines. While inhibition of perforin-mediated killing also inhibits NK-mediated cytotoxicity (Smyth *et al.*, 1999), complete inhibition is achieved only with the combination of anti-TRAIL and anti-FasL antagonistic antibodies (Takeda *et al.*, 2001). Endogenously produced interferon-γ plays a critical role in inducing Apo2L/TRAIL expression on NK cells and T cells

(Kayagaki *et al.*, 1999). These findings suggest that Apo2L/TRAIL and FasL may contribute to the natural suppression of tumors by NK cells. Expression of FasL on cells other than NK cells might also contribute to tumor suppression (Owen-Schaub *et al.*, 1998).

7.2 Involvement of death receptors in response of tumor cells to anticancer therapy

The response of cancer cells to chemotherapeutic agents and γ-radiation involves induction of apoptosis in response to the inflicted cellular damage (Rich *et al.*, 2000). The ability of anticancer agents to induce tumor-cell apoptosis is influenced by a host of oncogenes and tumor suppressor genes that regulate cell-cycle checkpoints and death-signaling pathways. The *p53* tumor suppressor gene is a key determinant of these responses (el Deiry, 1998; Kirsch and Kastan, 1998). Phosphorylation-induced stabilization of p53 in response to cellular damage plays a pivotal role in mediating cell-cycle arrest as well as apoptosis. The particular response elicited by p53 depends on the cell type and context, as well as the presence of coexisting genetic aberrations. The induction of apoptosis by p53 involves multiple and apparently redundant mechanisms (Schuler and Green, 2001). p53 can directly activate the mitochondrial death pathway by inducing the expression of specific target genes, such as *Noxa* (Oda *et al.*, 2000), *PUMA* (Nakano and Vousden, 2001), or *Bax* (Miyashita and Reed, 1995). p53 may also promote cell death by inhibiting the transcriptional activity of NF-κB (Ravi *et al.*, 1998b; Wadgaonkar *et al.*, 1999; Webster and Perkins, 1999), thereby repressing NF-κB-dependent expression of a host of survival genes. These observations indicate that p53-induced death is mediated by multiple redundant pathways leading to mitochondrial activation of Apaf-1/caspase-9. In support of this notion, cells from caspase-$9^{-/-}$ mice are highly resistant to chemotherapeutic drugs and irradiation (Kuida *et al.*, 1998a[Q1]). Accordingly, inhibition of this mitochondrial pathway by overexpression of Bcl-2 (Strasser *et al.*, 1994) or inactivation of *Bax* (Zhang, L. *et al.*, 2000) can also render tumor cells resistant to anticancer drugs or γ-radiation.

DNA damage also promotes the expression of the death receptors, CD95 (Muller *et al.*, 1998) and DR5/TRAIL-R2 (Wu, G.S. *et al.*, 1997). Although p53 promotes their DNA damage-induced expression, death receptors are not essential for DNA damage-induced apoptosis. Cells from CD95-deficient (*lpr*), CD95L-deficient (*gld*), $FADD^{-/-}$, or caspase-$8^{-/-}$ mice are resistant to death receptor-induced signals, but remain sensitive to chemotherapy and irradiation-induced apoptosis (Eischen *et al.*, 1997; Fuchs *et al.*, 1997; Newton and Strasser, 2000). Likewise, overexpression of FLIP prevents tumor-cell apoptosis by death receptors, but not by chemotherapeutic agents or γ-radiation (Kataoka *et al.*, 1998). Conversely, cells from $p53^{-/-}$ mice resist DNA damage-induced apoptosis, but remain normally susceptible to CD95-induced death, and p53-deficient tumor cells can be killed with CD95L or Apo2L/TRAIL (Fuchs *et al.*, 1997; Ravi *et al.*, 2001). These findings indicate that the death receptor and DNA damage/stress-induced signaling pathways operate largely independently until they converge at the level of mitochondrial disruption (*Figure 3*).

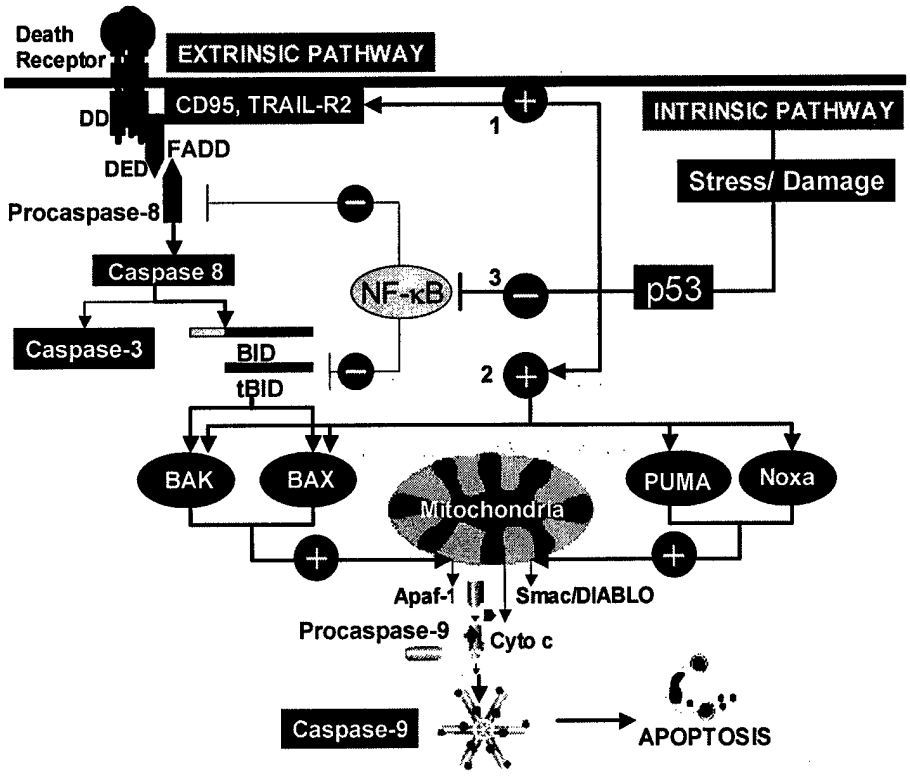

Figure 3. Cross-talk between the death receptor (extrinsic) and stress-induced (intrinsic/mitochondrial) death signaling pathways.

Cellular stress or DNA damage results in stabilization of p53. p53 can promote mitochondrial activation of caspase-9/Apaf-1 by inducing the expression of specific target genes, such as *Noxa*, *PUMA*, or *Bax*. The stress/DNA damage and death receptor signaling pathways operate largely independently until they converge at the level of mitochondrial disruption. The following mechanistic links between the intrinsic and extrinsic pathways may account for the synergistic cytotoxicity of chemotherapeutic agents/irradiation and death ligands: (i) DNA damage promotes expression of the death receptors (CD95 and DR5/TRAIL-R2). (ii) Cellular damage inflicted by chemotherapeutic agents can promote expression of BAK. (iii) p53 may inhibit the transcriptional activity of NF-κB.

Although death receptors play contributory, yet dispensable, roles in the response to conventional chemotherapy or irradiation, death receptor–ligand interactions may be instrumental for the action of cancer immunotherapy or specific anticancer agents. One example of how anticancer agents may employ death receptors-ligands in their actions is all-*trans*-retinoic acid (ATRA), a retinoid that induces complete remissions in approximately 10% of patients with acute promyelocytic leukemia. ATRA induces APL-cell differentiation followed by postmaturation apoptosis through induction of Apo2L/TRAIL (Altucci *et al.*, 2001). ATRA-induced expression of Apo2L/TRAIL is associated with activation of NF-κB-induced antiapoptotic target genes (Altucci *et al.*, 2001). Since the majority of patients with APL are resistant to ATRA, and complete remission induced by

ATRA is followed by the emergence of ATRA-resistant disease, it will be interesting to determine whether ATRA-resistant APL cells can be treated by inhibition of NF-κB and/or administration of Apo2L/TRAIL.

7.3 Targeting death receptors for treatment of cancers

Genetic aberrations that render cells resistant to diverse chemotherapeutic agents or ionizing radiation, such as loss of the *p53* tumor suppressor gene or overexpression of Bcl-2, underlie the observed resistance of human cancers to conventional anticancer therapy (Kaufmann and Earnshaw, 2000). Identifying approaches to induce apoptosis in tumors that harbor such genetic impediments could lead to effective therapeutic interventions against resistant human cancers. Since death receptors provide an alternative mechanism of activating caspases and triggering cell death, ligand- or antibody-induced engagement of death receptors may be an attractive strategy for anticancer therapy. However, the clinical utility and therapeutic ratio of this approach depends on the differential sensitivity of tumor cells and normal tissues to each agent and/or the ability to target delivery of death ligands/antibodies to tumor cells. Although TNF-α and CD95L can induce apoptosis of several types of tumor cells *in vitro*, their clinical application in cancer therapy is hindered by the serious toxicity of these ligands *in vivo*. Systemic administration of TNF-α causes a serious inflammatory septic shock-like syndrome that is induced by NF-κB-mediated expression of proinflammatory genes in macrophages and T cells. Systemic administration of FasL or agonistic antibodies against CD95 causes lethal hepatic apoptosis. In contrast to these ligands, Apo2L/TRAIL holds enormous promise for anticancer therapy.

A broad spectrum of human cancer cell lines express death receptors for Apo2L/TRAIL (TRAIL-R1/DR4 and TRAIL-R2/DR5) and exhibit variable sensitivity to Apo2L/TRAIL-induced apoptosis (Ashkenazi *et al.*, 1999). Although Bcl-2 protects cells from diverse cytotoxic insults, Bcl-2 overexpression does not confer significant protection against induction of apoptosis by Apo2L/TRAIL (Ravi *et al.*, 2001). Likewise, tumor cells that resist DNA damage-induced apoptosis by virtue of loss of p53 also remain susceptible to induction of apoptosis by TRAIL/Apo2L. Cancer cells with wild-type p53 ($p53^{+/+}$) and their isogenic $p53^{-/-}$ derivatives generated by deletion of p53 via targeted homologous recombination are equally sensitive to TRAIL/Apo2L-induced apoptosis (Ravi and Bedi, 2002). The tumoricidal activity of Apo2L/TRAIL *in vivo* has been confirmed in preclinical animal models (athymic nude or SCID mice) carrying human tumor xenografts without any evidence of toxicity to normal tissues (Ashkenazi *et al.*, 1999; Roth *et al.*, 1999; Walczak *et al.*, 1999). Apo2L/TRAIL prevented the growth of evolving breast or colon or glial cancers after xenotransplantation, and decreased the size of established tumors. More importantly, systemic treatment with Apo2L/TRAIL significantly improved the survival of tumor-bearing animals without any deleterious effects on normal tissues. Likewise, monoclonal antibodies that engage the human Apo2L/TRAIL receptors, DR4 and DR5, also demonstrate potent antitumor activity without any evidence of toxicity (Chuntharapai *et al.*, 2001; Ichikawa *et al.*, 2001).

An especially encouraging feature of Apo2L/TRAIL or agonistic antibodies against death receptors for Apo2L/TRAIL is that they induce apoptosis of tumor cells while sparing normal tissues. While TRAIL-R1 and TRAIL-R2 are broadly expressed in most organ systems, normal cells frequently express two additional TRAIL receptors, TRAIL-R3 (TRID or DcR1) and TRAIL-R4 (TRUNDD or DcR2) that serve as decoys and confer protection against Apo2L/TRAIL-induced death. The expression of decoy receptors provides a potential molecular basis for the relative resistance of normal cells to Apo2L/TRAIL-induced death (Marsters *et al.*, 1999). Other studies indicate that high levels of FLIP may protect normal cells from death receptor-induced apoptosis (Kim, K. *et al.*, 2000). Regardless of the specific mechanism(s) involved, the differential sensitivity of tumor cells and normal tissues to Apo2L/TRAIL-induced cytotoxicity makes this ligand a promising investigational anticancer agent. One potential concern was raised by the reported ability of a polyhistidine-tagged recombinant version of human Apo2L/TRAIL (Apo2L/TRAIL.His) to induce apoptosis *in vitro* in isolated human hepatocytes (Jo *et al.*, 2000). This concern was alleviated by subsequent studies using a Zn-bound homotrimeric version of human Apo2L/TRAIL that lacks exogenous sequence tags (Apo2L/TRAIL.0), which has been developed as a candidate for human clinical trials (Lawrence *et al.*, 2001). These studies demonstrated that Apo2L/TRAIL retains potent antitumor activity but is non-toxic to human or nonhuman primate hepatocytes *in vitro*. Moreover, intravenous administration of Apo2L/TRAIL in cynomolgus monkeys or chimpanzees was well-tolerated with no evidence of changes in liver enzyme activities, bilirubin, serum albumin, coagulation parameters, or liver histology (Lawrence *et al.*, 2001).

Although Apo2L/TRAIL can induce apoptosis independently of p53 or Bcl-2, cancer cell lines exhibit a wide heterogeneity in their sensitivity to Apo2L/TRAIL *in vitro*, and certain lines are resistant to this ligand (Ashkenazi *et al.*, 1999). As might be appreciated from the earlier discussion of the molecular determinants and regulators of death receptor-induced apoptosis, cancer cells may evade Apo2L/TRAIL-mediated apoptosis by mutational inactivation of death-signaling genes or aberrant expression of proteins that block death-signaling pathways. *Bax* is a frequent target of mutational inactivation in human cancers that harbor mutations in genes that govern DNA mismatch repair (MMR) (approximately 15% of human colon, endometrial, and gastric carcinomas). More than 50% of MMR-deficient colon adenocarcinomas contain somatic frame-shift mutations in an unstable tract of eight deoxyguanosines in the third coding exon (spanning codons 38–41)[$(G)_8$] within Bax (Rampino *et al.*, 1997). In addition, a similar frame-shift mutation results from loss of a G residue from a repetitive sequence in the second exon (LeBlanc *et al.*, 2002). MMR-deficient human colon carcinoma cells are rendered completely resistant to Apo2L/TRAIL by inactivation of *Bax* (Deng *et al.*, 2002; LeBlanc *et al.*, 2002; Ravi and Bedi, 2002). While both $Bax^{+/-}$ and $Bax^{-/-}$ sister clones activated apical death receptor signals, including activation of caspase-8 and cleavage of BID, Apo2L/TRAIL could not induce mitochondrial disruption or cell death in $Bax^{-/-}$ tumor cells. These data suggest that the basal expression of BAK in tumor cells may not be sufficient to substitute for BAX in mediating death receptor-induced apoptosis. In addition to loss of BAX, cancer cells may also evade

Apo2L/TRAIL-induced death by overexpression of the caspase-8 inhibitor, FLIP, or upregulation of Bcl-x_L (Burns and el Deiry, 2001). As both FLIP and Bcl-x_L are NF-κB-inducible proteins, this mechanism of resistance may operate in cancers that have constitutively high NF-κB activity (Ravi *et al.*, 2001). Since NF-κB is frequently activated by diverse genetic aberrations, growth factors, cytokines, viral proteins, costimulatory interactions, and stressful stimuli in diverse cancer types, it may be a common denominator of the resistance of many human cancers to Apo2L/TRAIL. Conversely, such cancers may be sensitized to Apo2L/TRAIL-induced death by inhibitors of NF-κB (Ravi and Bedi, 2002). Tumor cells can also be sensitized to Apo2L/TRAIL-induced apoptosis by various chemotherapeutic agents or ionizing radiation. Since death-receptor and DNA damage/stress-induced pathways operate largely independently until they converge at the level of mitochondrial disruption, the simultaneous delivery of both signals may have synergistic cytotoxicity. Moreover, conventional chemotherapeutic agents may also potentiate Apo2L/TRAIL-induced tumor cell death by upregulating p53, DR5/TRAIL-R2, and BAK (LeBlanc *et al.*, 2002). Although the combination of Apo2L/TRAIL with either NF-κB inhibitors or conventional anticancer agents may exert synergistic antitumor effects, additional studies are required to evaluate and optimize the safety and therapeutic ratio of such regimens *in vivo*.

8. Conclusion

The last decade has witnessed breathtaking progress in our understanding of cell death and its fundamental physiologic importance in multicellular animals. Enormous strides have been made in identifying the molecular assassins and the mechanisms by which they direct cell death. As our knowledge of death receptors and their signaling pathways has grown, so too has our appreciation of the key survival signals that keep them in check. It is now evident that evolution has designed an intricate molecular circuitry that maintains a dynamic balance between death receptors and antiapoptotic proteins. The stringent regulation of death receptor-induced apoptosis enables signal-dependent induction of physiologic cell death while protecting the organism from the devastating consequences of unscheduled or uncontrolled apoptosis. These insights into the molecular regulation of cell death have opened exciting avenues for therapeutic interventions against diseases that involve too much apoptosis or the failure of physiologic cell death. The challenge before us is to design innovative therapeutic strategies that counteract these defects by targeting death receptors or their regulatory pathways. Apo2L/TRAIL may prove that we have already embarked on a journey from death receptors to successful anticancer therapy. The next decade will test whether our investment in defining the basic mechanisms of cell death will harvest rich dividends in diverse fields of clinical medicine.

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